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THE RELATIONSHIP BETWEEN TENDON MORPHOLOGY AND FUNCTION

By

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SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
INSTITUTE OF ORTHOPAEDICS & MUSCULOSKELETAL SCIENCE

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ABSTRACT

Tendons have specific functions which are important in the role they play in locomotion. These specific functions are also important to consider when reconstructing or repairing damaged tendons to ensure restoration of normal physiological characteristics. The aim of this project is to correlate molecular and morphological characteristics of tendons with their mechanical properties which relate to physiological function. The results showed that the elastic modulus (material stiffness) was significantly different for the different tendons and ligaments in the distal part of the equine forelimb. The elastic modulus showed a significant positive correlation with the mass average collagen fibril diameter (MAD) for the different structures. The superficial digital flexor tendon (SDFT) showed a wide range of elastic moduli values in different animals and these showed a significant positive correlation with the MAD in the SDFT. The matrix composition also correlated with material properties; water content showed a significant negative correlation with elastic modulus and a significant positive correlation with glycosaminoglycan (GAG) content. Thus tendons composed of a stiffer material have larger collagen fibril diameters which are associated with lower water and GAG contents. These characteristics should be considered when choosing suitable replacements in tendon reconstruction procedures.

Epidemiological studies have shown that the incidence of partial rupture to the SDFT increases with increasing horse age. Correspondingly, age related changes were found in the properties of the equine SDFT. Elastic modulus decreased significantly with increasing horse age and both fascicle cross sectional area and fibril diameters decreased significantly with increasing age in the central core of the SDFT, the site where degeneration and subsequent injury most commonly occurs. Thus, as the horse ages it shows an increase in the proportion of small diameter collagen fibrils and a reduction in elastic modulus (material stiffness) in the SDFT which may predispose the tendon to injury.

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DEDICATION

This thesis is dedicated in memory of my colleague and friend Mike Kayser from the Electron Microscope Unit who unexpectedly passed away before the completion of this work. I will always have lots of good memories of working in the laboratory with Mike as he always made electron microscopy interesting with his great sense of humour and he is sadly missed.

DEFINITIONS

Structural Properties	Units	Definition
Ultimate Load	kN	Maximum load at failure.
Structural Stiffness	N/mm	Gradient of the load/deformation curve.
Material Properties		
Elastic Modulus (material stiffness)	MPa	Gradient of the linear region of the stress-strain curve.
Stress	MPa	Load/cross-sectional area.
Ultimate stress (strength)	MPa	Load at failure divided by the cross-sectional area.
Strain	%	Percentage specimen elongation.
Ultimate strain	%	Percentage tendon deformation recorded at peak failure.
Collagen Fibril Diameters		
Collagen fibril index (CFI)	%	Percentage of area covered by collagen; therefore is a measure of the collagen to noncollagen ratio in the extracellular matrix.
Mass average collagen fibril diameter (MAD)	nm	Mean of the diameter distribution; if each diameter value is plotted against the percentage of total fibril area that is occupied by fibrils of that diameter.

ABBREVIATIONS

ACL	-	Anterior Cruciate Ligament
CDET	-	Common Digital Extensor Tendon
CFI	-	Collagen Fibril Index
CO_2	-	Carbon Dioxide
COMP	-	Cartilage Oligomeric Matrix Protein
CS	-	Chondroitin Sulphate
CSA	-	Cross-Sectional Area
DDFT	-	Deep Digital Flexor Tendon
DS	-	Dermatan Sulphate
DNA	-	Deoxyribonucleic Acid
ECM	-	Extracellular Matrix
GAG	-	Glycosaminoglycan
HA	-	Hyaluronic Acid
HCL	-	Hydrochloric Acid
H&E	-	Haemotoxylin and Eosin
Hz	-	Hertz
kN	-	Kilo Newton
LDET	-	Lateral Digital Extensor Tendon
LP	-	Lateral Peripheral
MAD	-	Mass Average Collagen Fibril Diameter
MP	-	Medial Peripheral
MPa	-	Megapascals
MRI	-	Magnetic Resonance Imaging
MSC	-	Mesenchymal Stem Cells
N	-	Newton
PG	-	Proteoglycan
PSGAG	-	Polysulphated Glycosaminoglycan
SDFT	-	Superficial Digital Flexor Tendon
SEM	-	Scanning Electron Microscopy
SL	-	Suspensory Ligament
TEM	-	Transmission Electron Microscopy
UV	-	Ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Tendons provide a link between muscle and bone and they function to transmit the force generated by muscular contraction. Tendons have one of the highest tensile strengths of any soft tissue in the body. There are two reasons for this; firstly, the main constituent of tendons is collagen which is one of the strongest fibrous proteins; secondly, the collagen fibres are arranged parallel to the direction of tensile force. Tendon fulfils its primary role as a flexible force-transmitting element very effectively (Evans & Barbenel, 1975) and yet failure of this passive tissue leads to great disability.

Different tendons have specific functions, which are optimised for the role they play in locomotion. Identification of these specific functions in relation to tendon structural and material properties is important in terms of understanding the requirements for tendon reconstruction and repair, in restoration of appropriate physiological function. Tendons vary markedly in design, most likely related to their specific functions. Tendons have a range of shapes and sizes; they may also vary in thickness along their length. For example, the human Achilles tendon is very short in comparison to the equine superficial digital flexor tendon (SDFT). Some tendons have a flatter shape than others such as the equine common digital extensor tendon (CDET) and some tendons also have to go around corners, such as the long head of the biceps tendon. A high proportion of injuries sustained in athletic and exercise related activities affect specific tendons and these have commonality in terms of energy storing function. However, it is important to know how the properties of energy storing tendons compare to tendons that rarely sustain injury in order to identify reasons why they are more susceptible to damage.

The physiological function of the tendon depends on the mechanical properties of the structure. These properties are also governed by the composition of the matrix and organisation of the matrix components, all of which are controlled by the cells, which respond to stimuli. Therefore, the functional input appears to control the tendon structure. The optimisation of material properties such as strength and stiffness are essential for a tendon to function efficiently. Strength and stiffness can be defined by measuring the tendon as a whole structure (structural stiffness and ultimate load), or by

considering the material properties (elastic modulus and ultimate stress) of the tendon and its architecture (i.e. arrangement of material). Consideration of both material and structure is essential in understanding the structure-function relationships in different tendons. This thesis postulates that tendon morphology determines function. Tendons with different functional roles will be investigated to determine how specific functional behaviour relates to both mechanical properties and morphological features.

Chapter one presents a review of the current literature relating to tendon structure, mechanical properties and function with particular emphasis on equine forelimb digital flexor tendons and the human Achilles tendon. The equine superficial digital flexor tendon (SDFT) and the human Achilles tendon have many similarities, in that they fulfil similar functional roles in contribution to energetic efficiency of locomotion, acting as energy storing tendons and both sustain a high incidence of injury. Thus the horse provides an ideal "natural" animal model to study the pathophysiology of tendon injury in the human Achilles tendon.

1.2 TENDON INJURIES

Many racehorses and other competition horses are lost from training each year due to partial rupture of the flexor tendons, in particular the SDFT. A recent epidemiological survey of Thoroughbred (an equine athlete specifically bred for racing) injuries and fatalities at British racecourses found that 46 % of forelimb injuries involved the flexor tendons or suspensory ligaments (Williams *et al.*, 2001). In another epidemiological study, Pickersgill (2000) has shown a 43 % incidence of ultrasonographically-detectable tendon lesions in horses in training. Injury of the flexor tendons in the horse results in a high degree of morbidity, with prolonged periods out of work and a considerable risk of recurrent injury once athletic activity has been resumed (Goodship, 1993). SDFT injuries heal slowly, with 20-60 % of affected horses returning successfully to racing but with up to 80 % of horses sustaining reinjury (Sawdon *et al.*, 1996; Silver *et al.*, 1983). It has been documented that most tendon lesions develop in the central region of the SDFT (Marr *et al.*, 1993). The factors leading to tendon injury are not well understood

although fatigue, lack of fitness, poor conformation, systemic disease and incoordinate action are all recognised as predisposing to tendon rupture (Webbon, 1973).

A similar injury involving partial rupture is also seen in the Achilles tendon of human athletes involved in competitive sports (Smart *et al.*, 1980) as well as those in recreational sport and day-to-day activities (Hess *et al.*, 1989). Rupture of the Achilles tendon often results in months off training and sometimes even a permanent end to strenuous exercises that involve running or jumping. In human runners, 6.5-18 % of all overuse injuries are to the Achilles tendon (Clement *et al.*, 1981). The number and incidence of tendon injuries in general have increased substantially during the last few decades and it is estimated that tendon injuries account for 30-50 % of all injuries related to sports (Kannus, 1997).

1.3 FUNCTIONS OF TENDON

Several functions have been attributed to tendon in addition to its major role as a force transmitter. Tendons can function as power amplifiers during rapid muscle contraction (Hill, 1951), acting similar to the way in which a catapult works. A catapult relies on sudden release of constraining force and elastic recoil. A catapult that has been stretched slowly can recoil rapidly. The work returned in the recoil cannot exceed the work done to stretch the catapult, but it is done in a shorter time so the power is greater. Elastic mechanisms in some jumping insects can act as muscle power amplifiers by directly storing the work done by the muscle shortening as elastic energy within "tendons" and then releasing it rapidly (Bennet-Clark, 1975; Gronenberg, 1996). More recently, models of human athletes have demonstrated that increasing the series elasticity increases muscle work required in a single jump because elastic mechanisms allow the muscle to operate at lower than average shortening velocities and higher work outputs (Bobbert, 2001).

1.3.1 Energy-storing Tendons

Tendons can act as elastic energy stores. This function provides an important mechanism for increasing the energetic efficiency of muscular activity (Alexander, 1984 & 1988; Cavagna *et al.*, 1977). The elastic properties of tendon are important in running (Alexander, 1984) and have implications for the control of movement (Rack & Ross, 1984). In specific tendons, for example, the human Achilles tendon and equine SDFT, the elasticity of the tendon is utilised as a means of elastic energy storage to optimise the energetic efficiency of locomotion. Essentially the tendon acts in a manner analogous to the spring in a pogo stick. Other studies have also demonstrated that the digital flexor tendons of pigs are of a design well suited to enable them to act as elastic energy stores (Shadwick, 1990). At maturity, the pig digital flexor tendons have twice the tensile strength and elastic modulus but only half the strain energy dissipation of the corresponding extensor tendons. In the horse, at the gallop, as the forelimb contacts the ground the fetlock is hyper-extended, the flexor tendons are placed under very high tensile loads and strains almost to failure **(Figure 1.1)**. The SDFT recoils in the swing phase of locomotion, flexing the metacarpophalangeal (MCP) joint, as the leg is then propelled forward ready for the next stride. To maximize energy storage the tendons elongate to a point close to failure. It is thought that this may account for the relatively high incidence of injury during athletic activity. Functional tensile strains in equine flexor tendons have been recorded *in vivo* using small implanted Hall effect transducers. Such studies suggest tensile strains of around 3 % at the walk, 6-8 % at the trot and 12-16 % at the gallop (Stephens *et al.*, 1989). Wilson *et al.* (2003) have demonstrated that a catapult action for rapid limb protraction exists in the horse. It has been shown that horses cannot achieve the high power output required for rapid limb protraction by simple muscle contraction and they instead deploy an elastic biceps muscle to store and then release bursts of energy – this muscle catapult action has an output that is comparable to over 100 times its mass of non-elastic muscle. Therefore, the energy bursts from a horse's elastic bicep muscle provide power for rapid forelimb protraction in a flat-out gallop.

The ability of some tendons, e.g. energy storing tendons, to retain stored energy is a very important functional role. Tendon has low energy dissipation, returning in an elastic recoil approximately 93 % of the work previously done stretching it, and dissipating only 7 % as heat (Alexander, 2002). For example, in the horse, the forelimb SDFT can store up to 95 % of kinetic energy (Minetti *et al.*, 1999; Riemersma & Schamhardt 1985). The ability of a tendon to store energy is related to the amount by which it extends, which is often referred to as strain. However, it remains unclear whether the energy storing capacity of tendons can be reduced experimentally, and whether this compromises locomotor efficiency.

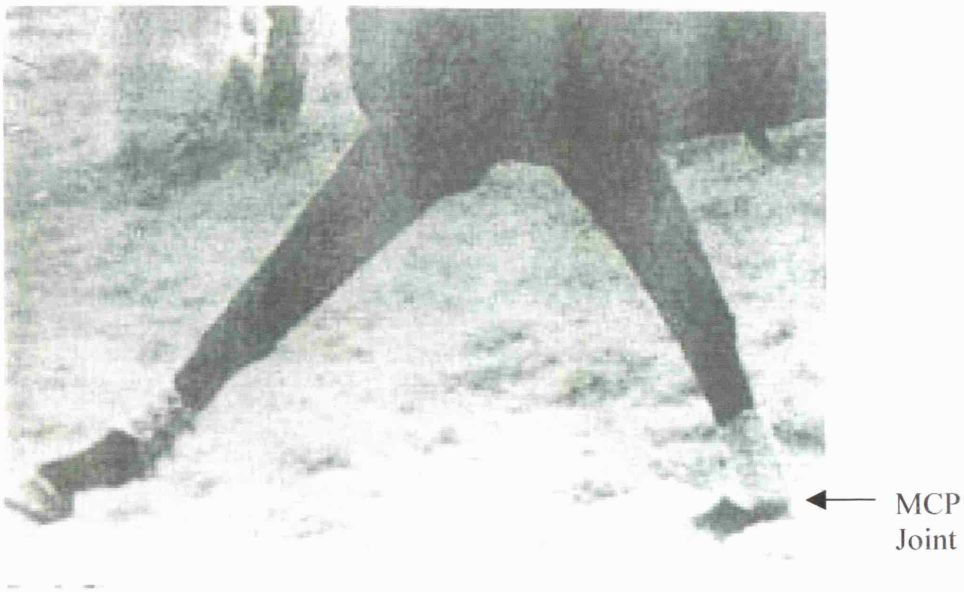


Figure 1.1: Hyperextension of metacarpophalangeal (MCP) joint (Goodship, 1993; with permission).

The ability to attenuate the forces produced by rapid and unexpected movement (Barnett *et al.*, 1961; Smith, 1954) is also dependent on tendon extensibility. This is because the compliance of a tendon enables it to act as a mechanical buffer to protect muscle fibres from damage during eccentric contractions (Griffiths, 1991). Thus, although in the

Achilles tendon of man this function is doubtful (except in the case of contracted and exhausted muscle) it may have relevance to the horse (Evans & Barbenel, 1975). Wilson *et al.* (2001) have shown that the function of muscle to dampen tendon vibrations is important in the horse. *In vivo* the SDFT is part of a passive spring apparatus, preventing hyperextension of the metacarpophalangeal joint; it is loaded via the accessory ligament with the almost redundant muscle acting merely to dampen the vibrations in the limb, following impact with the ground.

Apart from the role of tendons in energy storage, the muscle may also be contributing to the storage and recovery of elastic strain energy. Because the muscle is composed of both muscle fibres and tendinous materials, all these structures must be collectively 'tuned' to the spring properties for the muscle/tendon system to store and recover elastic strain energy during locomotion.

1.3.2 Positional Tendons

Positional locomotor tendons, can also be referred to as non-energy storing tendons. The primary role of these tendons is to transfer the tensile force created by muscle to a distal bone resulting in movement of a limb segment across a joint **(Figure 1.1)**. An example of a positional tendon is the equine CDET, which transmits muscle force to extend the limb prior to hoof placement. In the human leg, the tibialis anterior tendon plays a similar functional role in locomotion to the equine CDET. These tendons rarely sustain injury (Batson *et al.*, 2003; Goodship & Birch, 2001) and experience much lower peak strains, recorded to be 2.6 % at the trot in the sheep forelimb lateral digital extensor tendon (Kear & Smith, 1975), compared to weight-bearing tendons.

1.4 BIOMECHANICAL PROPERTIES

An understanding of the biomechanics of locomotion is a prerequisite to defining the functional roles of specific tendons. The complexity and functional roles of tendon can be appreciated through the mechanical properties of the tissue, although properties such

as morphology and matrix composition are also important. Early studies by Elliot (1965), defined the physical characteristics of tendon tissue to include high tensile strength, flexibility, and almost perfect elasticity.

1.4.1 Structural Properties Versus Material Properties

The main function of tendons is to transmit tensile load. The goal of mechanically testing tendons is to acquire load/deformation plots from which stress-strain curves **(Figure 1.2)** and hence material properties (e.g. elastic modulus, ultimate stress, ultimate strain) of the tendon tissue can be determined. Structural properties (e.g. load, deformation, structural stiffness, ultimate load) are also important, as they are measurements of the mechanical behaviour of the tendon.

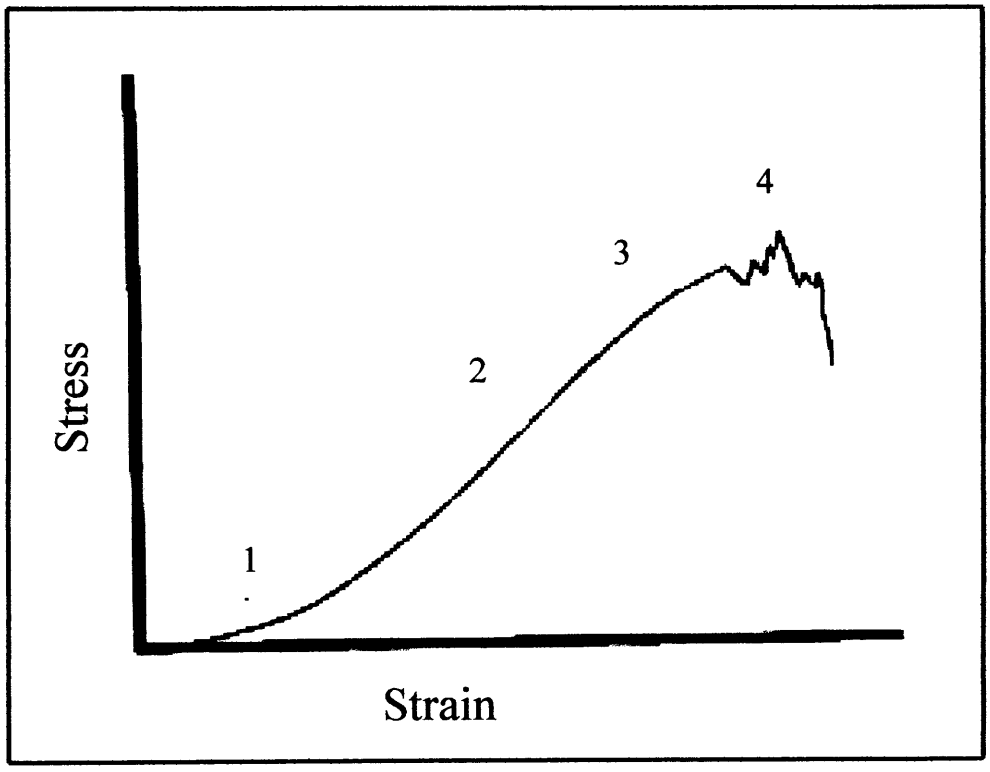


Figure 1.2: Diagrammatic representation of a stress-strain curve for loading of a tendon (Goodship *et al.*, 1994; with permission). Key: 1 = 'toe' region, 2 = linear region, 3 = yield, 4 = tendon rupture.

1.4.2 Stress-Strain Curves

Figure 1.2 gives a diagrammatic representation of a stress-strain curve for the tensile loading of a tendon to failure. The stress-strain behaviour of tendon is reported to comprise an initial non-linear region, with a transition to a linear region, leading to a quasi-linear relationship. However, at even higher stresses the tissue "yields" and so the entire stress-strain relationship is best described as sigmoidal (Evans & Barbenel, 1975). Other authors have also shown that the *in vitro* mechanical properties of tendon approximate a sigmoidal curve when force is plotted against elongation (Crevier *et al.*, 1996; Wilson & Goodship, 1990). The basic form of the quasi-static stress-strain relation for tendon was established some seventy years ago (Gratz & Blackberg, 1935).

The initial non-linear portion of the stress-strain curve is termed the 'toe' region. The elongation in this region is believed to be the result of straightening of the crimp, so that the relaxed collagen fibres become straighter as loading progresses (Abrahams, 1967; Diamant *et al.*, 1972; Elliot, 1965; Gustavson, 1956; Hirsch, 1974; Viidik *et al.*, 1965; Viidik, 1966; 1967a). In the toe region, relatively little force is required to elongate the tissue initially. As loading continues, the stiffness of the tissue increases and progressively greater force is required to produce equivalent amounts of elongation. The elongation is often expressed as strain, which is the deformation of the tissue calculated as a percentage of the original length of the specimen (Carlstedt & Nordin, 1989). The end of the toe region, which has been termed "toe limit strain", has been reported to have a strain value of between 1.5 and 4.0 % (Abrahams, 1967; Cronkite, 1936; Diamant *et al.*, 1972; Haut & Littel, 1972; Rigby *et al.*, 1959; Viidik, 1973).

The second region of the curve shows a linear response of the tissue to further elongation due to an increase in load. The gradient of this linear region defines the elastic modulus. Within the linear region of the curve the tendon recovers, when the load is removed. It behaves in an elastic manner. However, if a tendon is loaded and unloaded in a cyclical fashion some energy is lost in the form of heat just as it would occur when a rubber band is stretched repeatedly (Goodship, 1993).

The third region of the curve represents the yield point. At higher stresses, a yield point is reached beyond which the deformation at this point is not recoverable and is termed 'plastic deformation', indicating damage to the structure. The fourth region of the curve represents tendon rupture. A further increase in applied stress results in continued deformation to the point of complete mechanical failure, and this point is termed the ultimate tensile stress (Wilmink *et al.*, 1992).

Cyclical loading of tendons, *in vitro*, using a materials testing machine over a period of time shows 'preconditioning'. Preconditioning is shown as a shift of the load/deformation plot to the right with each successive loading cycle until eventually a steady state is reached **(Figure 1.3).** The behaviour of the tissue therefore becomes more repeatable. It is thought that this may have *in vivo* relevance in relation to 'warming up' prior to peak athletic performance, due to the fact that the mechanical behaviour of tendons changes during initial cyclical loading. There is evidence that the modulus of the tendon is reduced once a steady state is reached (Grieshaber & Faust, 1992; Schatzmann *et al.*, 1998). It also appears that the toe region is affected by preconditioning in that it becomes less pronounced. In addition, the plot of loading followed by unloading of the tendon forms a loop, starting and finishing at the same point. The loop, termed hysteresis, is formed as a result of the elastic recoverability of the tendon being less than 100 %. The area of the loop represents a loss of energy, largely in the form of heat that occurs during stretch and release of the tendon (Goodship & Birch, 2001).

Several studies (Batson *et al.*, 2003; Bennett *et al.*, 1986; Birch *et al.*, 2001; Ker, 1981; Shadwick, 1990; Woo *et al.*, 1982) have been carried out to investigate the mechanical properties of different tendons. These will be discussed in detail in **Chapter 5** (section 5.1.2 - Mechanical properties of tendons with specific functions).

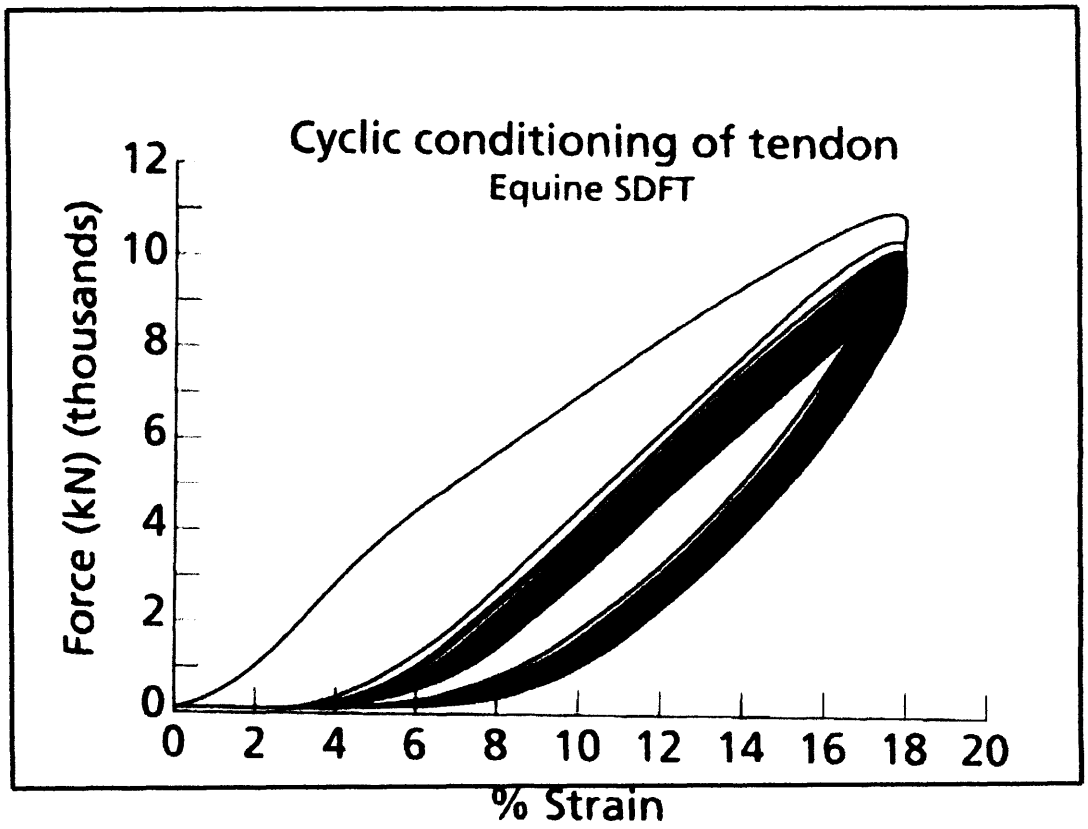


Figure 1.3: Cyclical loading of a tendon within the elastic range, showing hysteresis due to energy loss and preconditioning to a steady state (Goodship & Birch, 2001; with permission).

1.5 HIERARCHICAL STRUCTURE OF TENDON

Mammalian tendons have a complex hierarchical structure from the level of the collagen molecules to the gross entity and comprise a series of progressively larger subunits (Kastelic *et al.*, 1978). **Figure 1.4** gives a diagrammatic representation of the structural hierarchy of tendon. Paratenon is the loose connective tissue that surrounds tendons and allows movement of the tendon against surrounding tissues. This movement is assisted by synovial fluid, which is extruded from the parietal synovial membrane and from the visceral synovial membrane (epitenon). Under the paratenon, a fine connective sheath called epitenon, which is a relatively dense network of collagen fibrils, surrounds the

entire tendon. Collections of fascicles form the whole tendon and are wrapped up in the epitenon (Chowdhury *et al.,* 1991).

The endotenon is a thin reticular network of connective tissue inside the tendon that binds collagen fibres together. Along with its important function of binding, the endotenon network allows the fibre groups to glide on each other and carries blood vessels, nerves, and lymphatics to the deeper portion of the tendon (Elliot 1965; Hess *et al.*, 1989). The fibre bundles and fascicles are enclosed in endotenon allowing them to slide relative to one another, which contributes to overall flexibility (Benjamin & Ralphs, 1997).

Collagen fibrils are grouped to form a collagen fibre, which is the smallest tendon unit visible using light microscopy and is aligned from end to end in a tendon (Curwin, 1997). Collagen fibrils are orientated not only longitudinally but also transversely and horizontally, with the longitudinal fibrils also crossing each other forming spirals and plaits (Jozsa *et al.,* 1991). The fibres are collected into fibre bundles (subfascicles), and a group of subfascicles then forms a fascicle. One fascicle usually has three or four subfascicles, although Kastelic *et al.* (1978) reported that tendon fascicles may have up to 10-12 subfascicles. The number of subfascicles appears to vary from tendon to tendon, and occasionally even within the same tendon (Jozsa & Kannus, 1997).

Tendons are associated with muscles and form a continuation of the collagenous framework that supports the contractile muscle fibres. The endomysia and perimysia, and epimysia, to some extent, are reflected in the tendon distal to the musculotendinous junction (Goodship *et al.*, 1994).

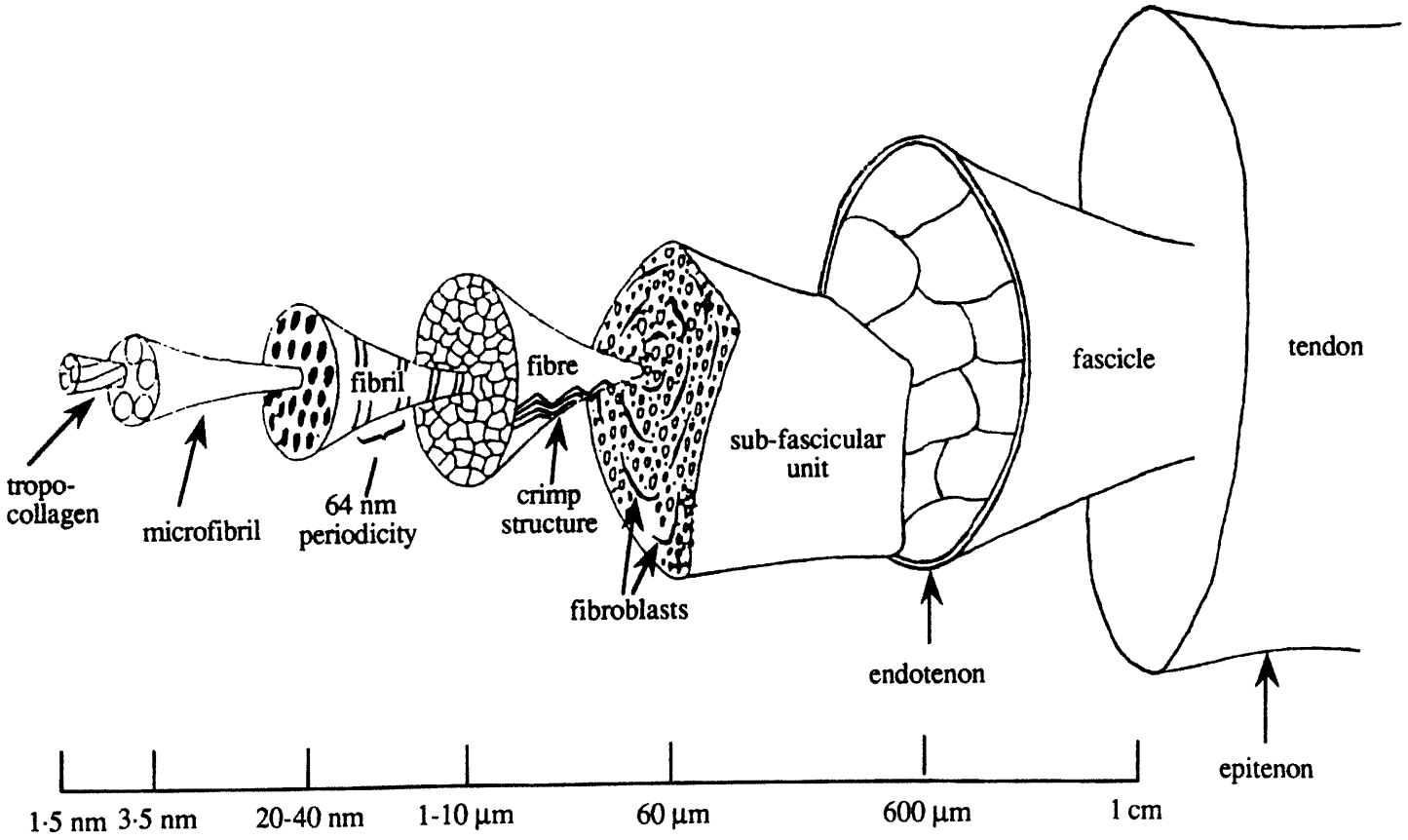


Figure 1.4: Structural hierarchy of tendon (Adapted from Kastelic *et al.*, 1978).

1.5.1 Tendon Cells

The majority of cells in tendons are of fibroblast phenotype (Kjaer, 2004). Mast cells, endothelial cells and axons are known to be present as well (Hart *et al.*, 1995). Tendon cells are also sometimes referred to as tenocytes. This is a common synonym for a tendon fibroblast (Benjamin & Ralphs, 2000). The immature form of a tenocyte is a 'tenoblast' (Jozsa & Kannus, 1997). Tendon cells have attracted relatively little interest because attention has focused on the collagen that accounts for the mechanical properties of tendons. However, McNeilly *et al.* (1996) have shown that tendon fibroblasts have an elaborate shape. They lie in longitudinal rows and have numerous sheet-like cell processes that extend into the extracellular matrix (ECM). The processes surround bundles of collagen fibres and come into contact with processes from cells in adjacent rows. Benjamin & Ralphs, (1997) suggest that the cells form a three dimensional communicating network that extends throughout the tendon and could form the basis of an important load-sensing system that allows a tendon or ligament to modulate the composition of its ECM in response to changes in loading pattern.

Goodship *et al.* (1994) showed that within the tenocyte population, at least three apparently distinct morphological cell types could be observed in longitudinal section, when stained with Haemotoxylin and Eosin (H & E):

(1) cells with flattened, cigar-shaped, dense nuclei,
(2) cells located in linear groupings, with more rounded, dense nuclei,
(3) those of an immature nature, with less dense, open nuclei.

More recently, Chuen *et al.* (2004) have also identified both round tendon cells and elongated tendon cells in healthy adult human patellar tendon in longitudinal section using H & E staining **(Figure 1.5)**.

These different cell types have not yet been characterised in terms of surface markers or gene expression for specific matrix proteins, nor is it known whether they have specific

roles in tendon metabolism or merely represent different stages in the maturation of tenocytes.

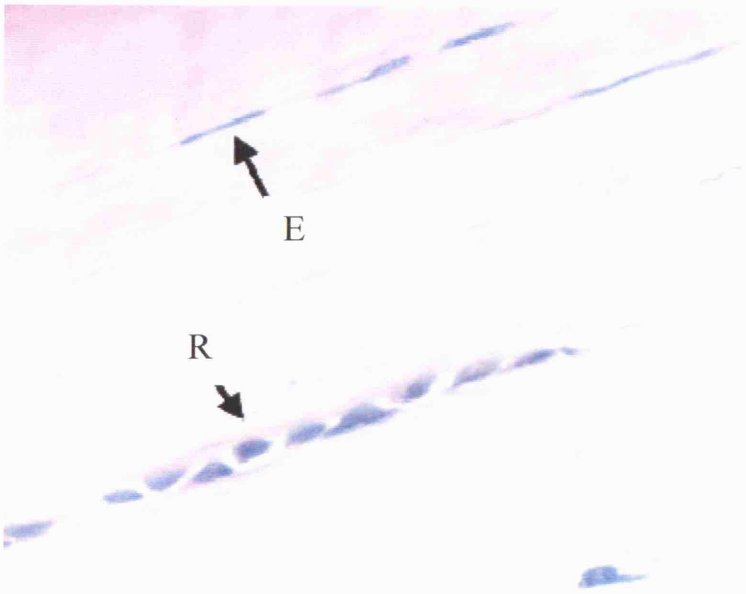


Figure 1.5: Tendon in longitudinal section. Round tendon cells (R) and elongated tendon cells (E) are identified in healthy human patellar tendon (Haematoxylin and Eosin, x 400) (Adapted from Chuen *et al.*, 2004).

1.5.2 Matrix Composition

Tendons consist of interdependent aggregations of collagen, elastin, proteoglycans (PGs), glycolipids, water and cells. Tendon tissue is formed by an intricate network of macromolecules constituting the extracellular matrix which interact with tendon cells. Collagen and non-collagenous glycoproteins, which are the major constituents of the extracellular matrix, play a pivotal role in accomplishing the unique function of tendon (Cherdchutham *et al.*, 1999). The interaction between these constituents has an important function in conducting and withstanding the tensile load of tendon (Cribb & Scott, 1995).

1.5.2.1 Collagen

Collagen can be found in both fibril and non-fibril forming forms. Fibril forming collagens are distinct from other proteins in that the molecule comprises three polypeptide chains, which form a unique triple-helical structure. For the three chains to wind into a triple helix they must have the smallest amino acid, glycine at every third residue along each chain. Each of the three chains therefore has the repeating structure Gly-Xaa-Yaa, in which Xaa and Yaa can be any distinct amino acid but are frequently the imino acids proline and hydroxyproline. More than 20 genetically distinct collagens exist in animal tissues (Kadler *et al.*, 1996). In tendon however, roughly 70-80% of the dry weight of normal tendon is composed of type I collagen (Woo *et al.*, 2000a). Bone and skin are also composed of type I collagen (Woo *et al.*, 2000a). The fibril forming collagens provide the structural framework of tissues. Type I collagen forms the principal tensile-resistant fibre, but smaller quantities of types III, V and VI are also present in tendon (Waggett *et al.*, 1996). In addition, some type II collagen is present in areas of tendon under compression; where tendons wrap around bony or fibrous pulleys, and bony attachments (Benjamin & Ralphs, 1998).

1.5.2.2 Collagen Cross-linking

Covalent crosslinks between collagen molecules are formed by two important processes, which are the enzyme-mediated extracellular post-translational modification process and nonenzymatic glycation (Eyre, 1987). Hydroxylysylpyridinoline and lysylpyridinoline crosslinks, which tie the adjacent collagen molecules together, are the end products of the hydroxylysine aldehyde pathway, one of the post-translational modifications of collagen. Pentosidine crosslinks are the result of the reaction of reducing sugars with protein, and have been qualified as the irreversible amino acid or advanced glycation end products (AGEs) (Last *et al.*, 1990). This is a non-enzymatic, time-dependent process. Therefore, this type of crosslink accumulates in the tissue after maturity and can be used as an indicator to assess the remodelling rate of the collagen network in tendon (Cherdchutham *et al.*, 1999). Collagen crosslinks are needed for the stability of collagen fibrils and are essential to fulfil the physiological functions of this type of tissue

(Tsuzaki *et al.*, 1993). Lysyl oxidase plays an important role in the formation and repair of the extracellular matrix by oxidising lysine residues in collagen, thereby initiating the formation of covalent crosslinkages (Kagan & Li, 2003).

1.5.2.3 Collagen Type

Several tissues with different collagen fibril diameters and orientations and hence mechanical properties contain both type I and III collagen e.g. tendon, skin, aorta and amnion (Keene *et al.*, 1987). The interrelationships of these molecules are presently unknown, and there is limited information available on the features that characterise the different collagen types. Type I and III collagen molecules show a remarkable homology to each other (Miller, 1985) and both form fibrils with the same periodic D-banding (Adachi & Hayashi, 1985). The difference in mechanical properties, therefore, in tissues with different ratios of type I and III collagen is thought to be due to the way in which the two collagens form fibrils. There is evidence that the pN- and pC- forms of both types I and III collagens are involved in fibrillogenesis. pN-type I and pN-type III collagens are present on fibrils of small diameter, but absent from fibrils of larger diameter (Fleischmajer *et al.*, 1981). It is currently thought that the aminopropeptides of procollagen molecules are cleaved after they are released from the cell and before fibril formation. The further observation that the amino propeptide of pN-type III collagen is excised far more slowly than the equivalent peptide of pN-type I collagen under physiological growth conditions suggests that the limitation to fibre growth may be at least partially related to the proteolytic excision of the type III amino propeptide (Fessler *et al,* 1981). These observations suggest that the amino and carboxyl propeptides of both types I and III collagens may be directly involved in regulating fibril growth. The arrangement of type I and III collagen in fibrils however is not completely understood. There is presently limited information available on the regulation of type I fibres in specific tendons i.e. energy storing and positional. Studies by Keene *et al.* (1987) have found that fibrils in skin, tendon and amnion are copolymers of both type I and III collagen. However it remains unclear how these are formed and the influence these have on fibril diameter regulation, and also in which tendons this occurs.

1.5.2.4 Elastin

Elastin is a structural, fibrous protein which constitutes approximately 1-2 % of the dry weight of tendon (Kannus, 2000). Elastin fibres consist of a central core of elastin and a peripheral layer composed of a loose arrangement of microfibrils (Parry *et al.*, 1978b). The hydrophobic properties of elastin are considered to provide the protein with the ability to undergo elastic extension and subsequent recoil, which also explains the considerable extensibility of tissues like the arterial vessels, lungs and skin compared to tendon (Bailey, 2001; Martinez-Hernandez & Amenta, 1990). The function of elastin is not well understood and it is unclear whether it plays a role in the material or structural characteristics of tendon. It is also unclear what the mechanisms are for its formation and degradation in relation to the properties of tendon.

1.5.2.5 Water

Tendon consists of 65-75 % water and much of this is associated with proteoglycans in the extracellular matrix (Akeson *et al.*, 1984). Together, water and proteoglycans have important lubricating and spacing roles in tendons that allow collagen fibres to glide over each other (Amiel *et al.*, 1995). Limited information is available on these interactions, or more importantly in terms of the control of water content in specific tendons, and also about the role in relation to specific material and structural properties of specific tendons.

1.5.2.6 Proteoglycans and Glycosaminoglycans

Proteoglycans comprise approximately 1 % of the tendon matrix (Robbins & Vogel, 1994). They are large (molecular weight 10^6 Da) negatively charged hydrophillic molecules, composed of a protein core in which one or more glycosaminoglycan (GAG) chains are covalently attached. The protein core binds to a specific site on a collagen fibril and the GAG chains hold the fibrils at defined distances from each other (Scott, 1995).

The predominant proteoglycan found in the region of tendon subjected to tensional loading is decorin, which may be glycosylated with either a dermatan sulphate (DS) or chondroitin sulphate (CS) GAG chain. Tendons are not of uniform composition along their length. Differences in regions of tension and compression in terms of morphology and proteoglycan composition have been described for flexor tendons of the rabbit, dog, and cow (Breuer *et al.*, 1990; Gillard *et al.*, 1977; Koob & Vogel, 1987; Merrilees & Flint, 1980; Okuda *et al.*, 1987; Vogel & Koob, 1989; Vogel & Heinegard, 1985). In the region of each tendon that wraps around a bone and receives compressive in addition to tensional forces, the cells have a rounded morphology and there is an increased amount of GAG and increased amounts of large proteoglycan. In contrast, the cells in the tensional regions of the same tendon are elongate, the GAG content of the tissue is low, and the predominant proteoglycan is decorin (Vogel & Heinegard, 1985).

1.5.2.7 Non-collagenous Proteins

Tendon tissue contains various non-collagenous proteins whose identities and functions are not yet well established. Cartilage Oligomeric Matrix Protein (COMP) is a non-collagenous protein originally identified in articular cartilage, which has since been identified in tendons (Hedbom *et al.*, 1992; Smith *et al.*, 1995). Recently, a number of non-collagenous proteins have attracted interest because of their interactions with the fibrillar collagen network, other matrix components and tenocytes. These may play important roles in the organisation and function of the tendon extracellular matrix during development, homeostasis, adaptation and injury (Smith *et al.*, 1997). A number of proteoglycans – including the large aggregating proteoglycans and the small proteoglycans, decorin, biglycan and fibromodulin - have been investigated in tendon (Daniel & Mills, 1988; Evanko & Vogel, 1990; Vogel & Evanko, 1987; Vogel & Koob, 1989; Vogel *et al.*, 1993). These studies have shown that the non-collagenous extracellular matrix is not homogeneous along the length of the digital flexor tendons, reflecting the different biomechanical environments experienced in different regions of the tendon. The function of COMP is not clear yet, although it appears that it is

synthesised in response to, and is necessary for tendon to resist, load (Smith *et al.*, 1997).

1.5.3 Collagen Fibrils

Collagen fibrils are the units of tensile strength of the tendon (Patterson-Kane *et al.*, 1997a) and are cylindrical structures from which the diameters can be measured in transverse section, using electron microscope techniques **(Figure 1.6)**. Collagen fibril diameters vary greatly, and both within and between tendons, fibril diameters range from 40 – 500 nm. In the mature horse, the equine SDFT collagen fibrils may be classified as 'large' diameter, measuring up to 165-215 nm or 'small' diameter, measuring up to 35-40 nm in size (Parry *et al.*, 1978a). In the human Achilles tendon, the fibrils are between 30 nm and 130 nm in diameter (Kannus, 2000).

Collagen fibrils can also be visualized in longitudinal section using electron microscope techniques **(Figure 1.7)**, showing a banding pattern with a repeating periodicity every 62-67 nm; referred to as D-periodicity (Gillard *et al.*, 1977; Hulmes *et al.*, 1973; Kastelic *et al.*, 1978). The lengths of collagen fibrils are far less characterised due to basic difficulties in measurement on sectioned tissue (Birk *et al.*, 1997; Trotter & Wofsy, 1989) but entire fibrils with lengths in the range 1-100 μm have been isolated from embryonic chick tendon, skin and cornea (Birk *et al.*, 1995; 1996). However, the mechanism of fibril assembly in tissue and how fibril diameter and length are regulated is still unclear.

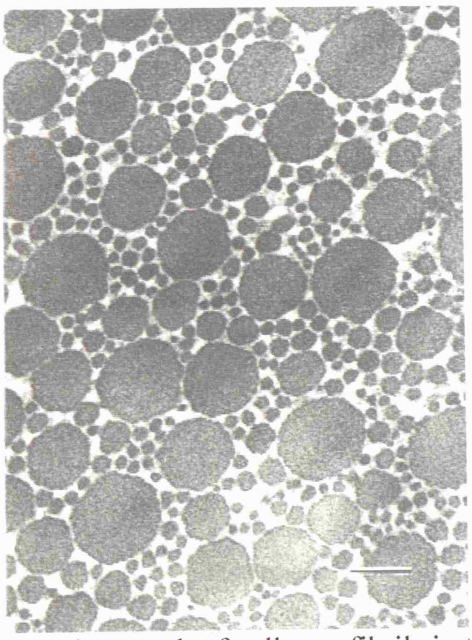


Figure 1.6: Electron micrograph of collagen fibrils in transverse section (Bar represents 200 nm).

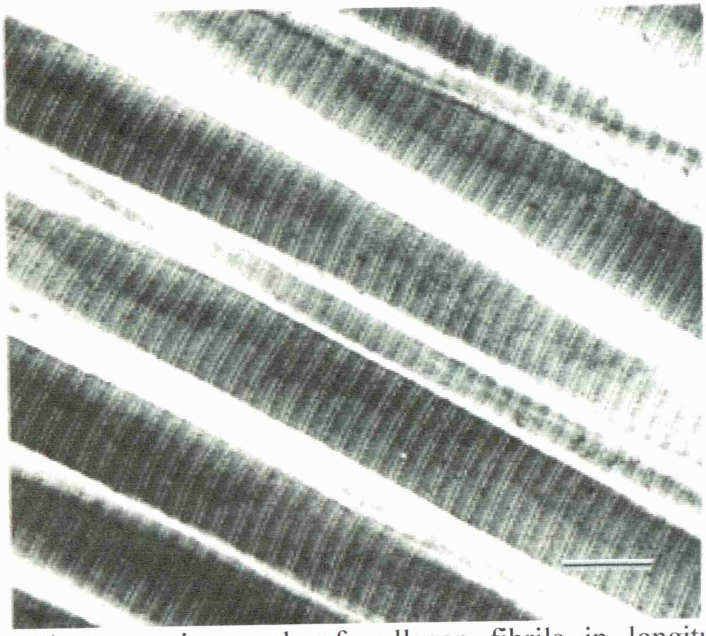


Figure 1.7: Electron micrograph of collagen fibrils in longitudinal section (Bar represents 280 nm).

1.5.3.1 Collagen Fibril Formation

Collagen fibril formation **(Figure 1.8)** is basically a self-assembly process (i.e. one which is to a large extent determined by the intrinsic properties of the collagen molecules themselves) but it is also sensitive to cell-mediated regulation particularly in young or healing tissues (Kadler *et al.*, 1996). Recent attention has been focused on 'early fibrils' or 'fibril segments' of 10 μm in length which appear to be intermediates in the formation of mature fibrils that can grow to be hundreds of micrometers in length. Holmes *et al.*, (1994) indicated that these early fibrils can be unipolar (with all molecules pointing in the same direction) or bipolar (in which the orientation of collagen molecules reverses at a single location along the fibril). However, the principles governing the self-assembly of unipolar and bipolar fibrils and how cells regulate this process remains unclear. The occurrence of such early fibrils has major implications for tissue morphogenesis and repair (Kadler *et al.*, 1996) if an understanding of the occurrence of bipolar fibrils in tissues and in healing wounds, and the identification of cell-mediated factors in determining collagen fibril size and shape can be determined.

In some tissues, such as the cornea and periodontal ligament, the fibrils are uniform in diameter, all being around 40 nm throughout life, and also have highly regulated orientations. In other collagenous structures, including tendon, the fibrils have a similar appearance early in development but with age, some fibrils have larger diameters and a bimodal distribution is seen (Goodship *et al.*, 1994). There is some evidence from *in vitro* studies that the maturation of the fibrils may be controlled by glycosaminoglycan (GAG) molecules. Experimental data suggest that high hyaluronate concentrations inhibit fibril aggregation and increasing the concentration of chondroitin sulphate removes the inhibitory influence (Parry, 1988).

Decorin, fibromodulin and lumican are members of the family of small leucine rich proteoglycans (SLRPS). They are all known to bind to the surface of collagen fibrils and the binding site for decorin has been shown to be distinct from that of fibromodulin (Hedbom & Heinegard, 1993; Svensson *et al.*, 2000). Each of these SLRPs are present

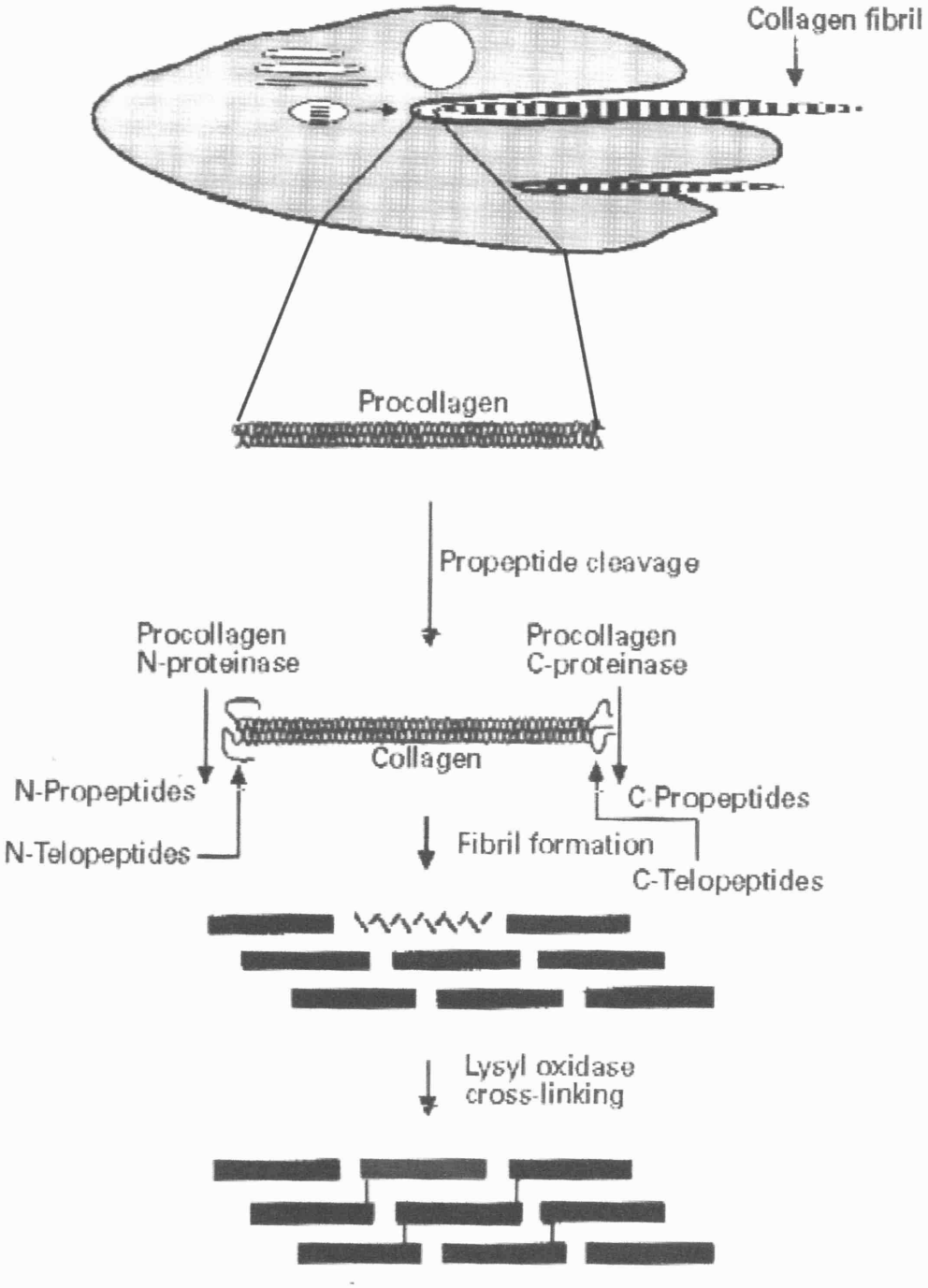


Figure 1.8: Extracellular events in the synthesis of fibrillar collagens (Adapted from Kadler *et al.*, 1996).

in embryonic tendon (Birk *et al.*, 1995; Ezura *et al.*, 2000). The phenotypes of decorin, fibromodulin, and lumican deficient mice indicate that these molecules regulate collagen fibril assembly and growth by preventing the aberrant lateral fusion of collagen fibrils in connective tissues such as tendon, skin and cornea (Chakravarti *et al.*, 2000; Danielson *et al.*, 1997; Ezura *et al.*, 2000; Svensson *et al.*, 1999).

1.5.3.2 Crimp

A striking feature of tendon is 'crimp' – a planar wave pattern of collagen fibrils seen in longitudinal section. It is commonly suggested that crimp provides a buffer allowing tendons to elongate without becoming damaged (Amiel *et al.*, 1995). Fibrils within a fascicle can have different crimp characteristics and each possesses a 'crimp structure' observable in the polarising microscope (Kastelic *et al.*, 1978). If sections of tendon are examined using a polarising light microscope, the collagen fibrils show a waveform (Abrahams, 1967; Rigby *et al.*, 1959; Viidik & Ekholm, 1968), which appears as an alternating light and dark cross banding **(Figure 1.9)**, in a planar zig-zag wave structure (Diamant *et al.*, 1972) and is seen at the level of banded fibrils. This appearance results from the fact that the fibrils are crimped in the relaxed state. The crimp is extremely sharp and is confined to within 1 – 2 D periods (Dlugosz *et al.*, 1978). The crimp may be characterized by measurement of its angle and periodicity **(Figure 1.10)**. Both the wavelength and angle of the crimp may vary between fibrils, which means that some fibrils straighten out before others as the tendon is stretched. Consequently, when a tendon is stretched to failure, a sequence of fibril failure occurs, resulting in a partial rupture, such as that seen in many cases of tendonitis (Goodship *et al.*, 1994). The crimped nature of collagen fibres determines the characteristics of the 'toe' region of the stress-strain curve for tendon. The presence of crimp reduces the stiffness of the tendon during initial loading and the most likely function of crimp is to decelerate rapid loading thus preventing damage (Gathercole & Keller, 1991).

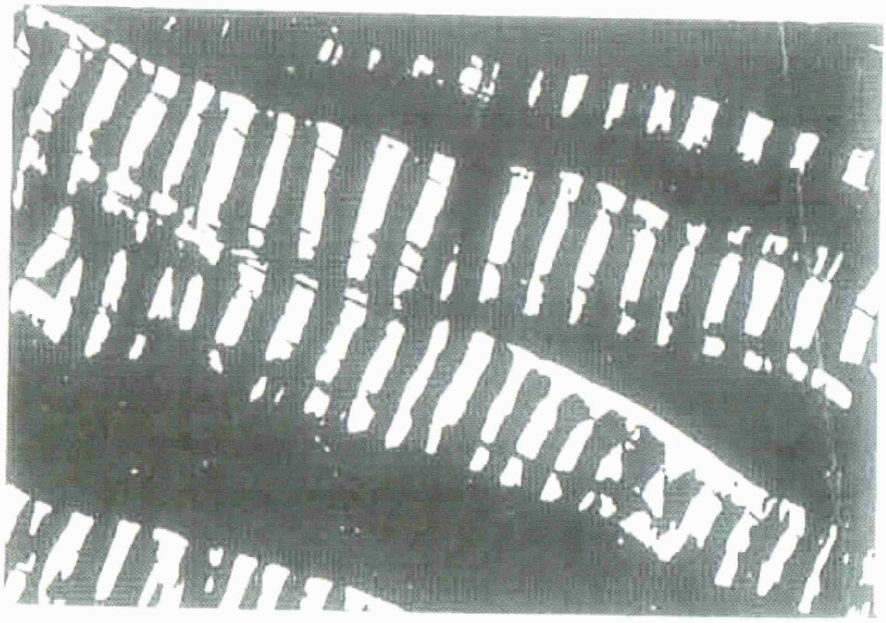


Figure 1.9: Demonstration of crimp in equine tendon (x220) (Wilmink *et al.,* 1992; with permission).

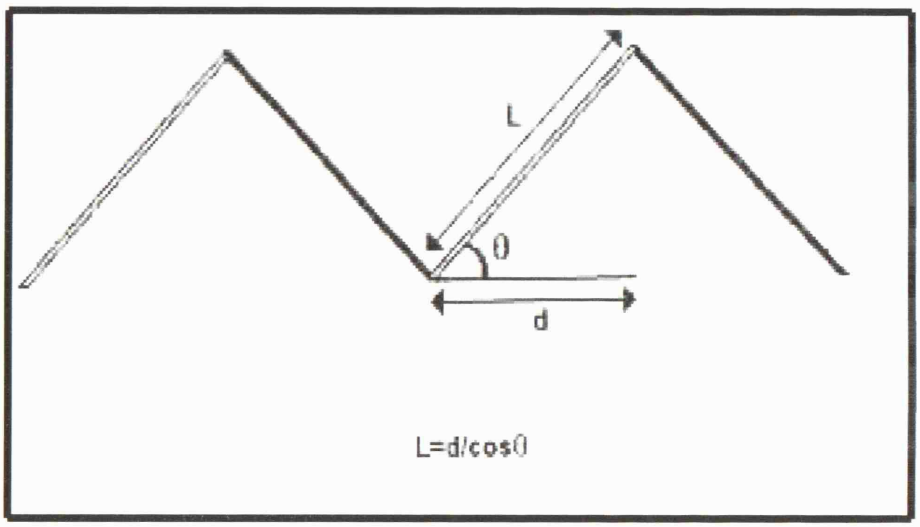


Figure 1.10: Definition of crimp angle and length measurements. L = true crimp length, d= measured crimp length, θ = crimp angle (Patterson-Kane *et al.,* 1997b; with permission).

1.5.4 Collagen Fibres

Collagen fibres are assembled from bundles of fibrils within a fascicle or sub-fascicle, and there is variation in the collagen fibre diameter in tendon. In the rat tail tendon, the diameter ranges from 5 – 30 μm (Angel & Georghe, 1985). In human tendons the fibre diameter can be as high as 300 μm (Elliot, 1965). Collagen fibres are visible under the scanning electron microscope (SEM) and lie parallel to each other, running in the longitudinal axis of the tendon **(Figure 1.11)**. Collagen fibres are also visible under the light microscope where lying between the collagen fibres and also running parallel to them are rows of tendon cells.

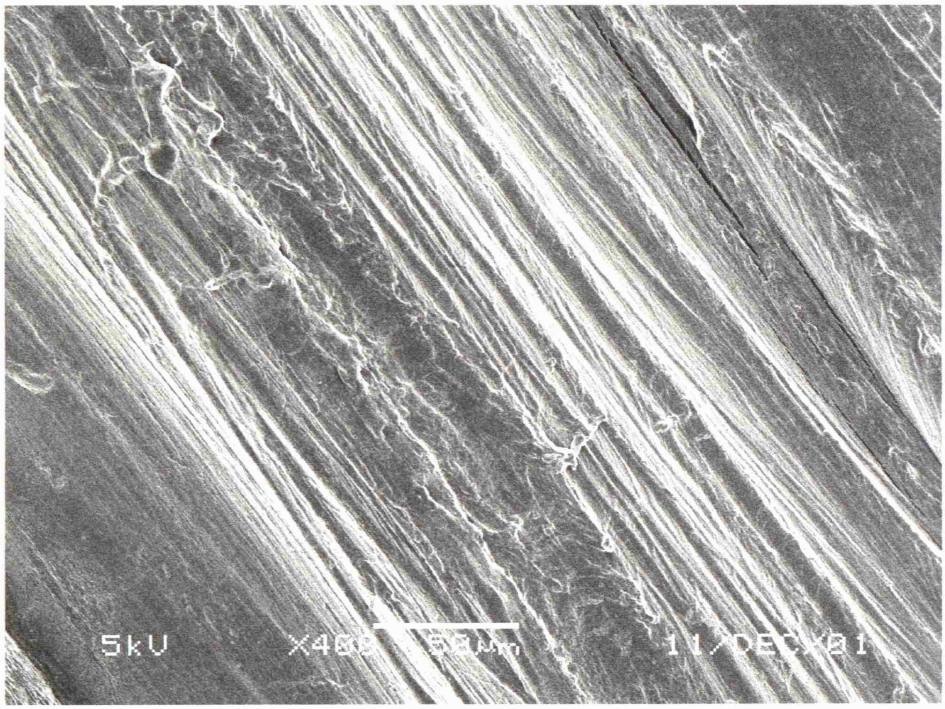


Figure 1.11: Scanning electron micrograph of SDFT in longitudinal section showing collagen fibres at the cut surface of a section at the mid-metacarpal level.

1.5.5 Blood Supply

The blood supply to the tendon is of great importance to supply nutrients and oxygen to the tissue and to remove carbon dioxide and lactic acid (Celli *et al.*, 1976; Potenza, 1976; Vailas *et al.* 1978). According to Benjamin & Ralphs (1997), in comparison with other tissues associated with synovial joints, tendons have a poor blood supply. It is much less than that of muscle, synovium or bone, but it is still important for normal function and for promoting healing. Blood flow is approximately 0.10 ml/g/mn in rabbit tendons compared with 0.27 ml/g/mn for muscle (White *et al.*, 1964). Blood supply increases in tendon with exercise and during healing (Backman *et al.*, 1991; Bray, 1995). Blood vessels in tendons form a conspicuous network in the epitenon from which longitudinal vessels run in the endotenon. As a result of the longitudinal excursion that tendons have to make within their sheaths, their blood vessels must be long and coiled. Not all parts of a tendon have a blood supply and regions subject to friction, compression or torsion are often avascular (Hergenroeder *et al.*, 1982; Kolts *et al.*, 1994; Lundborg *et al.*, 1997). Such areas are especially prone to tearing but the reason for this is unclear.

The flow of blood through tendons has been measured in several animal studies using washout technique. Stromberg (1971) obtained a value of 1.08 ± 0.42 ml/100g/min for the equine SDFT, but no significant increase in flow from exercise alone. Rates of 0.5 – 2.8 ml/100g/min have been recorded in other tendons from dogs, chickens and rabbits (Landi *et al.*, 1983; Piaggi & Mingione, 1981; Vailas *et al.*, 1978). Altered blood flow is often mentioned as a part of the aetiology of tendon injuries. Angiographic findings (Lagergren & Lindholm, 1958/59) have lead to suggestions that reduction in blood flow to a certain area of the Achilles tendon (2-6 cm above its insertion) might be a cause of injury (Smart *et al.*, 1980), as this region is the most common site of rupture. Reduced blood flow may diminish the capacity to supply nutrients and oxygen to a transiently weakened or injured structure after a loading session. Restoration of normal function after injury would depend largely on the vascular response (Weiland *et al.*, 1988). Blood flow has also been measured, using radiolabelled microspheres, in the patellar and Achilles tendons of the dog during running exercise (Bulow & Tondevold, 1982). There

was an increase in blood flow to both the patella and Achilles tendons during and after exercise.

The cyclical loading of tendons results in an associated ischemia during the period in which the tendon is under maximum tensile load. On relaxation a reperfusion occurs in which superoxides may be formed. Free oxygen radicals are toxic to tissues and cause local damage to cells thereby comprising matrix maintenance (Goodship *et al.*, 1994). In a study by Birch (1993), tenocytes cultured from explants of the central region of equine SDFT showed a significant decrease in proliferation when subjected to between 10^{-5} and 10^{-4} M hydrogen peroxide to stimulate a high free radical concentration. This experiment was then repeated, adding a free radical scavenger that protected the cells from the inhibitory effects of the free radicals. These studies suggest that agents acting as free radical scavengers used during intense exercise may have a role in reducing central core degeneration. However this would require further support from *in vivo* studies. Determining whether there is a link to the different structural features of specific tendons also remains unclear.

The vascular supply to the SDFT in the horse has been investigated by Stromberg (1973) and more recently by Kraus-Hansen *et al.* (1992). They documented, by use of contrast radiography, that the blood vessels of the SDFT are principally intratendinous and that the blood supply courses mainly in a longitudinal direction. The tendon is less well vascularised within its middle third, in the mid-metacarpal region (Fackelman, 1973; Stromberg, 1971). It has also been shown that the human Achilles tendon is poorly vascularised, particularly in its midportion, around 4cm above the calcaneus, and this is the site where rupture commonly occurs. It is thought that the decreased vascularity could affect the nutrition and remodelling potential of the tendon matrix in this region (Carr & Norris, 1989). It is not presently known which aspects of the matrix are affected and whether there is evidence in terms of collagen fibril diameters or biochemical changes. It also remains unclear to what extent the blood flow to connective tissue alters with mechanical loading of the tissue.

1.5.6 Effect of Matrix Composition on Biomechanical Properties

Some components of the extracellular matrix have been correlated to the biomechanical properties of tendon. There is a positive correlation between mass-average fibril diameter of the collagen fibrils and the ultimate tensile strength of tendon, skin and cartilage (Bailey *et al.,* 1970a; 1970b). It has also been shown in developing rat tail tendon, that material properties change (Haut, 1985) as mean fibril diameter increases (Parry & Craig, 1977; Scott *et al.,* 1981). However, there is one investigation (Bay *et al.,* 1993) where it was observed that collagen fibril diameters are not good predictors of elastic modulus in sheep anterior cruciate ligament (ACL). In a study by Parry *et al.* (1978b) it was shown that the mechanical properties of tendon are strongly correlated with the collagen fibril diameter distribution. In particular, it is postulated that the size distribution of the collagen fibrils is largely determined by two factors. First, if the collagen fibrils are to have a high tensile strength then they need to be large in order to maximize the density of intrafibrillar covalent crosslinkages. Consequently, large collagen fibrils are predicted to have a greater tensile strength than small diameter fibrils. Second, if the collagen fibril network is to return to its original form after compression or tension, the network must have sufficient interfibrillar crosslinks to inhibit non-recoverable creep. In addition to having the appropriate tensile strength, it is often important that a tissue is elastic and thus able to return precisely to its original length or shape after the removal of stress. For example, if the SL or SDFT of the horse or the Achilles tendon in man (these all being subjected to continuous long-term high stresses) were to suffer from non-recoverable creep, the functional role of the tissue would be in jeopardy. It is proposed that creep inhibition can be accomplished through the presence of small collagen fibrils (Parry *et al.,* 1978b). However, the reason for this remains unclear and it is also not presently known how this applies to tendons with different functions.

1.6 THE EFFECT OF AGEING ON TENDONS

Ageing has a considerable effect on tendon morphology. Maturation of tendons from the development of the newborn animal until skeletal maturity produces a series of well documented changes. Collagen fibril diameters are known to increase in both equine SDFT and human Achilles tendon changing from a unimodal distribution at birth, e.g. in foals (Batson, 2002), to a bimodal distribution at skeletal maturity (Parry *et al.,* 1978b). Webbon (1978) conducted a histological study of macroscopically normal equine digital flexor tendons. In the foetus, the SDFT and DDFT had some similar structural features, being composed of numerous longitudinally arranged cells with plump, granular nuclei (Holmes, 1971) between which lay eosinophilic (visible when stained) collagen fibres. As gestation progressed their cellular component was reduced as their fibrillar component increased. A reduction in the amplitude of the fibre waveform (i.e. "crimp"), which decreased with age was also seen in the SDFT and DDFT, which was reported to be a 'normal change'. The type of cells in a tendon, particularly the elongation of their nuclei (indicative of maturity), has been shown to depend on the tendon chosen (Holmes, 1971), its age, (Holmes, 1971; Inglemark, 1948) and the previous activity of the animal from which the tendon was taken (Inglemark, 1948). Plump nuclei of the tenoblast type have been seen in the SDFT from other species (Holmes, 1971) although their purpose is uncertain. Tenoblasts were found more frequently in forelimb tendons, possibly due to additional weight bearing, although mechanical work produces fewer, rather than more of such cells in experimental animals (Inglemark, 1948). Webbon (1978) suggested that it may be that the collagen in the forelimb SDFT at the metacarpal level of domesticated horses is subjected to stresses producing microtrauma which necessitates the presence of increased numbers of synthesising cells for replacement of the collagen. However, these early studies on tendon morphology are limited in that they do not provide any evidence of new collagen formation, making it difficult to draw conclusions on the effects of ageing on structure-function relationships in tendons with specific functions.

Ageing has an effect on fascicles and the material properties of tendon. Gillis *et al.* (1997) conducted a study of SDFTs from horses ranging in age from 2 to 23 years, which were not in active work. Results from this study showed that fascicle cross-sectional area (CSA) decreased significantly with increasing age and was significantly larger for 2 year olds than for older horses (>3 years old). This decrease in fascicle CSA with increasing age appears to represent increasing organisation within the tendon structure rather than change in tendon size with maturation, as no correlation was found between tendon CSA and age. It was also seen that the central region of SDFTs had larger fascicles than the peripheral region indicating that there may be a decreased stability between collagen fibre bundles and a correlation to fibre rupture in the central region. However, from this study it is not clear why this region does not re-organise into smaller fascicles. The factors controlling the size and number of fascicles have not been determined. In a further study by Gillis *et al.* (1995) on the same group of horses, it was shown that the elastic modulus increased with age, and was significantly greater for horses older than 2 years than for 2 year old horses. It is thought that the biomechanical changes detected with increasing age are the result of changes in tendon fibre organisation and increasing amounts of mature collagen crosslinks within the tendon over time. These horses were normal in terms of CSA of the tendons, therefore the effects of injury on mechanical properties and morphology were not investigated in this study. Studies in other species, including rat tail and canine tendons, have indicated that flexor tendon strength, energy conservation, and elastic modulus increase significantly during maturation and decrease from maturation to senescence (Danielsen & Andreassen, 1988; Elliot, 1965; Nathan *et al.*, 1978; Vogel, 1983; Walker *et al.*, 1976). It remains unclear how these changes relate to structure-function relationships in tendon and the mechanisms involved are presently unknown. However, it is important to determine when the change from maturity to senescence occurs in each of these species and how this point in time is defined so that different studies, and specific tendons can be compared.

Ageing of the extracellular matrix following the cessation of skeletal growth is much less understood. Collagen fibril diameters are known to decrease in the SDFT and suspensory ligament of the horse from maturity until senescence (Parry *et al.*, 1978a)

and also in rabbit Achilles tendons (Nakagawa *et al.*, 1994). In tendon it is thought that the collagen content remains unchanged, while the amount of proteoglycans and glycoproteins further declines with old age. The elastic components of tendon decrease and the water content of a tendon declines from 80-85 % at birth to approximately 30-70 % in old age (Hess *et al.*, 1989; Ippolito, 1986). With ageing, collagen becomes tougher, its tensile strength is reduced, its fibres shrink, and the tendon stiffens (O'Brien, 1992). With decreased proteoglycan and water content, this suggests less tendon elasticity. Furthermore, tendon blood flow and tenoblastic activity decrease with increasing age (Astrom & Westlin, 1994; Hastad *et al.*, 1958-59; Kannus & Jozsa, 1991). As a result of all these 'physiologic' age-related changes, an aged tendon is weaker than its younger counterpart due to structural damage to the tendon occurring from repetitive strain and loading, and is more likely to tear or suffer from overuse injury (Best & Garrett, 1994; Hess *et al.*, 1989; O'Brien, 1992). It is thought that tendinous tissue becomes fatigued as its reparative ability is overwhelmed by repetitive dysfunctional and microtraumatic processes, however the mechanisms for this are yet to be determined.

1.7 THE EFFECTS OF EXERCISE ON TENDONS

Studies have been carried out on a range of species to investigate the effects of exercise on tendons with most data coming from animal models. Most of the early studies have been carried out on Achilles tendons, i.e. energy storing, however, it would have been interesting to have also investigated tendons with different functions, e.g. positional tendons, to determine if these responded to exercise in a similar manner. In an early study, Inglemark (1948) compared collagen fibrils from the Achilles tendon of rats that had undergone 40 weeks of daily running to those of untrained rats. Electron microscopy was used to measure the thickness of fresh and OsO_4-impregnated collagen fibrils. Although the OsO_4-impregnated fibrils of trained animals were significantly thicker than those of untrained animals, this finding was not evident when comparing fresh collagen fibrils. More recently, Enwemeka *et al.* (1992) also studied the effect of exercise on the diameter of collagen fibrils taken from the Achilles tendon of rats.

Results showed an increase in collagen fibril diameter after just 10 weeks of exercise. However, no mechanical testing was performed in either of these experiments. Further studies that have examined mechanical changes of tendon in response to endurance training suggest that training results in increased tensile strength and stiffness. It has been shown by Viidik (1969) that a 40 week running regimen in rabbit Achilles tendons resulted in a higher ultimate tensile load and higher elastic modulus for the tendons from trained animals when compared to untrained controls. The limitations of this study, however, are in determining how these effects were mediated and whether an increase in stiffness in an energy storing tendon is a rational adaptation. It is also important to consider what conditions the controls were kept in, i.e. are caged rats really controls or are they an "underuse" model. Barfred (1971) found that the strength of the Achilles tendon was higher for wild rats, when compared to domesticated rats, suggesting that physical activity increases the strength of this tendon. Again these may have been caged rats in a disuse situation. Vilarta & Vidal (1989) also reported increased stiffness and tensile strength in the Achilles tendon of rats following a 30 day exercise programme. More recently, Simonsen *et al.* (1995) compared the adaptation response of the Achilles tendon of rats to strength training and a swimming (endurance) training regimen. Results showed that ultimate failure decreased with ageing, but that the age related decline was counteracted by the swim training. In contrast there was no effect as a result of strength training. However cross-sectional area of the tendon and mechanical properties were not measured, therefore it can not be concluded from this study whether the changes were structural or material in origin.

It might be expected that changes in a tendon's biomechanical properties may be associated with changes in collagen concentration. However, the majority of existing studies do not support this. Curwin *et al.* (1988) measured collagen concentration (collagen concentration has been consistently measured in terms of % of dry weight) in the Achilles tendon of growing chickens following an 8 week running programme. Results showed no difference in collagen concentration when compared to untrained chickens that were housed in a run (1.5 X 6.5 X 10 m). This study demonstrates that intensity of exercise and animal age may affect tendon adaptation to exercise and are

important variables to consider in studies involving treadmill running. A further understanding of connective tissue adaptation to exercise during growth is also required. Other studies have also reported that training does not increase collagen concentration in tendon (Vailas *et al.,* 1985; Viidik 1967b; Woo *et al.,* 1980).

The first study to examine tendons with different functions was carried out by Woo *et al.* (1980, 1981) who studied the effects of exercise on swine flexor and extensor tendons. It was found that swine digital extensor tendons underwent hypertrophy, along with an increase in modulus, cross-sectional area and a 22 % increase in tensile strength following long-term running exercise. In comparison, long-term running exercise resulted in no significant increase in the mechanical properties of the digital flexor tendon in the same animals. This study also indicated that the physical properties of the swine digital flexor tendons are different from those of the swine digital extensor tendon. It is thought that this difference in response to functional stress may be attributed to the biochemical composition and mechanical properties of these tendons that have different functions. In addition, Wood *et al.* (1988) observed in rat tendons that exercise induced an increase in the crimp angle of the collagen fibrils while the crimp length was decreased and that simultaneous treatment with anabolic steroids accentuated these changes. However, from this study it is difficult to conclude what the implications of these structural changes are on tendon function.

A number of exercise studies have also been carried out on equine tendons. Patterson-Kane *et al* (1997a) carried out a study to investigate the effect of exercise on equine tendons in relation to changes in the collagen fibril population. The study involved twelve Thoroughbred fillies, six of which underwent an 18 month treadmill training program involving galloping exercise (3 days/week, combined with exercise on a horse walker for 40 min for 6 days/week), while the remaining six control horses were exercised on a horse walker (6 days/week, for 40 min/day) over the same period. Results from this study showed that there is a regional change in fibril diameter distribution within the SDFT. The central core region exhibited an increase in the number of small diameter collagen fibrils compared with the fibrils in the peripheral

region of the tendon in horses that had not undergone high intensity exercise. Interestingly, there was no increase in cross-sectional area in these tendons, nor in collagen content or collagen linked fluorescence (an indicator of age of collagen) (Birch *et al.,* 1997). Also, the DDFT and SL did not exhibit any reduction in mass average diameter as a consequence of the imposed exercise, suggesting that the SDFT is preferentially loaded at a gallop, making it more prone to injury (Patterson-Kane *et al.,* 1998). Therefore, it appears that long periods of exercise, may dissemble the large diameter collagen fibrils rather than the change being due to new collagen replacing old. This may represent accumulation of micro-damage prior to overt clinical injury (Goodship & Birch, 2001). The mechanisms involved have yet to be determined but may result from increased levels of metalloproteinases. Birch *et al.* (1995) studied the GAG composition and fibril diameters in the SDFT of horses subject to an 18 month treadmill training programme. As a result of the exercise, the total sulphated GAG content in the central core of the tendon was significantly lower which may be due to a reduction in chondroitin sulphate, and the mass average fibril diameter was significantly lower. The authors suggested that the relative amount of GAGs play a role in mediating collagen fibril diameters in response to mechanical stimuli. However, the link between these molecules and fibrillogenesis may be important in understanding the effects of both age and exercise on tendon structure and mechanical properties.

It has also been shown that development of the equine SDFT may be influenced by the imposed level of exercise. In a study in Dutch Warmblood foals (Cherdchutham *et al.*, 2001), it was shown that the development of the SDFT in terms of collagen fibril diameter was related to the type of exercise. At age 5 months there was a significant decrease in fibril diameter in exercised and pasture-managed foals compared with the box rested group. The authors believe this increase in small diameter fibrils may be attributable to the formation of new fibrils rather than to the splitting or degeneration of old ones. However, it can not be concluded from this study whether these effects are long lasting, due to the fact that these foals were skeletally immature. Box rest was found to inhibit the development of tendon; however inappropriate levels of exercise

may damage the developing tendon, which has also been shown in rat (Vailas *et al.*, 1985) and chicken (Curwin *et al.*, 1988) studies.

1.8 TENDON DEGENERATION

It was first suggested by Forssell (1952) that a degenerative change in energy storing tendons precedes rupture. Kannus & Jozsa (1991) have also demonstrated that tendon rupture usually occurs as a result of an accumulation of degenerative change within the tendon. It is not presently known whether degenerative change preceding rupture is also true for positional tendons. Degenerative lesions are observed in both the equine SDFT (Webbon, 1977) and human Achilles tendons (Jozsa, *et al.*, 1989) and these lesions are usually located in the central core region **(Figure 1.12)**. Macroscopic examination of a transverse section from these tendons shows a pink or purple core. In the equine SDFT, the site most commonly affected by degeneration and subsequent rupture is the mid-metacarpal region (Webbon, 1977) where the cross-sectional area is the smallest (Crevier *et al.*, 1996; Smith *et al.*, 1994). In the human Achilles tendon, the site most commonly affected is 2-3 cm above the insertion onto the calcaneus. In a study by Schonbauer (1959) it was found that 53 % of human Achilles tendon ruptures occur usually 3 cm from the calcaneus, indicating site specific predisposition to this area.

Tendon degeneration involves histopathological alterations to cells, collagen fibres and non-collagenous matrix components. Histopathological studies have shown that ruptured Achilles tendons may include clear degenerative changes before the rupture, such as tissue necrosis, calcification (Jarvinen *et al.*, 2001), and angiofibroblastic hyperplasia, consisting of a proliferation of fibroblasts and new capillaries (Hyman & Rodeo, 2000). Structural alterations to collagen fibrils and fibres also occur as a consequence of tendon degeneration (Jozsa *et al.*, 1984, 1989), although it remains unclear how this is related to tendon structure-function interactions. The most frequent alterations are longitudinal splitting of collagen fibres and a decrease in the average collagen fibril diameter. In a study by Birch *et al.* (1998), on the matrix composition of degenerated tendons, it was found that the core region had an increased total sulphated

glycosaminoglycan content, a higher type III collagen, decreased collagen-linked fluorescence and higher cellularity. This is thought to be a result of a change in tenocyte metabolism but whether the stimulus for this was a natural age-related process, an exercise induced phenomenon or both, was uncertain. In human Achilles tendons, degenerate tendon tissue has been reported to contain hypoxic changes in mitochondria, endoplasmic reticulum, ribosomes, and the number of lysosomes in the tenocytes (Jozsa *et al.,* 1982). However, it also remains unclear the effect these have on structure-function interactions in tendon.

Recent studies have identified several factors that are probably important in the development of tendon degeneration. Tissue hypoxia caused by poor vascularity may result in impaired metabolic activity. Birch *et al.* (1997) have shown *in vitro* that oxygen-derived free radicals may injure a tendon during ischemia-reperfusion injury, which can occur because of local tendon hypoxia. Alternatively, exercise-induced hyperthermia has also been suggested as a possible cause of cell or tissue damage in the equine SDFT (Wilson & Goodship, 1994). In this study, probes were used to measure the temperature on the surface of the SDFT in an exercising horse. Peak temperatures in the core of the tendon were 43 – 45 °C, a level which is sufficient to cause fibroblast death in cells from other species *in vitro.* Damage to tendon cells could result in an inability to metabolise normal matrix components and therefore subsequent matrix degeneration (Fackelman, 1973). Cultured SDFT tenocytes are however significantly resistant to hyperthermia. It appears that the increase in temperature in the tendon core does not persist for long enough to cause cell death *in vivo,* although repeated exposure may compromise cell function resulting in an altered matrix composition (Birch *et al.,* 1997). These are all important studies reporting findings on how a tendon overuse injury develops. However, it is now important to determine the mechanism behind these findings concerning tendon degeneration. It is important to now link these changes in structure to the function of specific tendons.

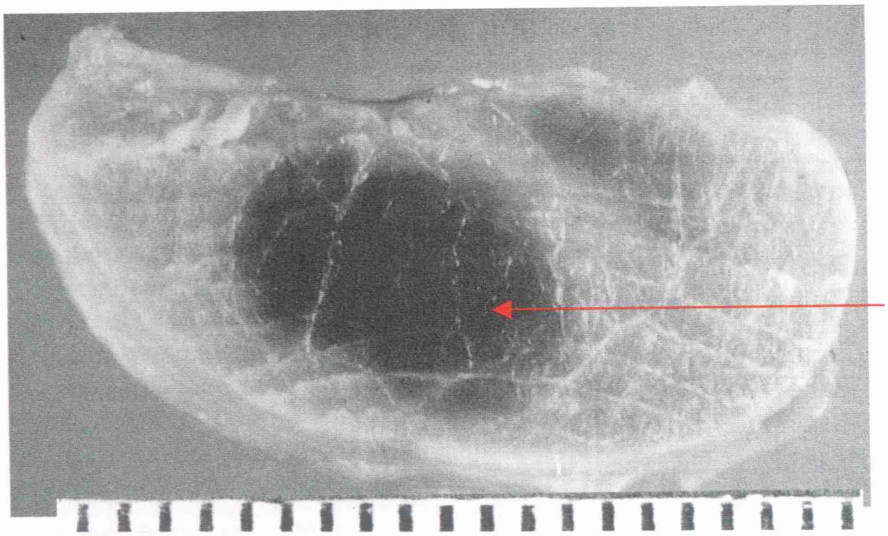


Figure 1.12: Degenerated SDFT showing central core lesion (Wilson & Goodship, 1994; with permission). Scale is in millimetres.

1.9 GROSS MORPHOLOGY

1.9.1 Tendons of the Equine Forelimb

In the distal part of the equine forelimb there are four major tendons; the superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), common digital extensor tendon (CDET), and the lateral digital extensor tendon (LDET). In addition, the suspensory ligament (SL), which is also known as the interosseous muscle and often referred to as a tendon of the equine forelimb, plays an important role in providing motion and stability to the musculoskeletal system.

The forelimbs carry 55-60 % of the horse's body weight at rest and also function as the principal shock absorbers necessary at faster gaits (Rooney, 1974). The tendons of the common and lateral digital extensors pass down the dorso-lateral aspect of the metacarpal bone, while those of the superficial and deep flexors are located on the palmar side of the metacarpal bone. The interosseous muscle (suspensory ligament) is

also situated on the palmar aspect, between the bone and the flexor tendons **(Figure 1.13)**.

1.9.1.1 Superficial and Deep Digital Flexor Tendons

The collagenous component of the SDFT and DDFT originates as a continuum of the connective tissue framework of the superficial digital flexor muscle and the deep digital flexor muscle respectively, which are located on the caudal aspect of the radius. The SDFT becomes subcutaneous after emerging from the carpal canal. It forms a sleeve around the DDFT at the level of the proximal sesamoid bones. The deep part of the sleeve splits opposite the middle of the proximal phalanx to allow the SDFT to attach to the distal tubercles of the proximal phalanx and the adjacent complementary fibrocartilage of the middle phalanx (Dyce *et al.,* 1987). The DDFT receives the very strong distal accessory (check) ligament at the mid-metacarpal level, which arises from the palmar carpal ligament. The tendon then passes the metacarpophalangeal joint in the sleeve formed by the SDFT, and beyond the middle of the proximal phalanx and over the middle phalanx. It then widens before passing over the distal sesamoid (navicular) bone to terminate on the distal phalanx (Dyce *et al.,* 1987).

Both the SDFT and DDFT are held in place by three retinacula, which are thickenings of the deep fascia. The first, the palmar retinacular ligament, arises from the abaxial borders of the proximal sesamoid bones. It adheres to the SDFT, thus restricting movement between the tendon and the sesamoids. The second retinacular ligament, the proximal digital retinacular ligament, resembles an X where the four corners attach near the proximal and distal tubercles of the proximal phalanx. The main body and the ligament fuses with the SDFT. The third, the distal digital retinacular ligament, arises from the medial and lateral borders of the proximal phalanx along with the abaxial palmar ligaments of the phalangeal joint. It fuses with the palmar surface of the DDFT.

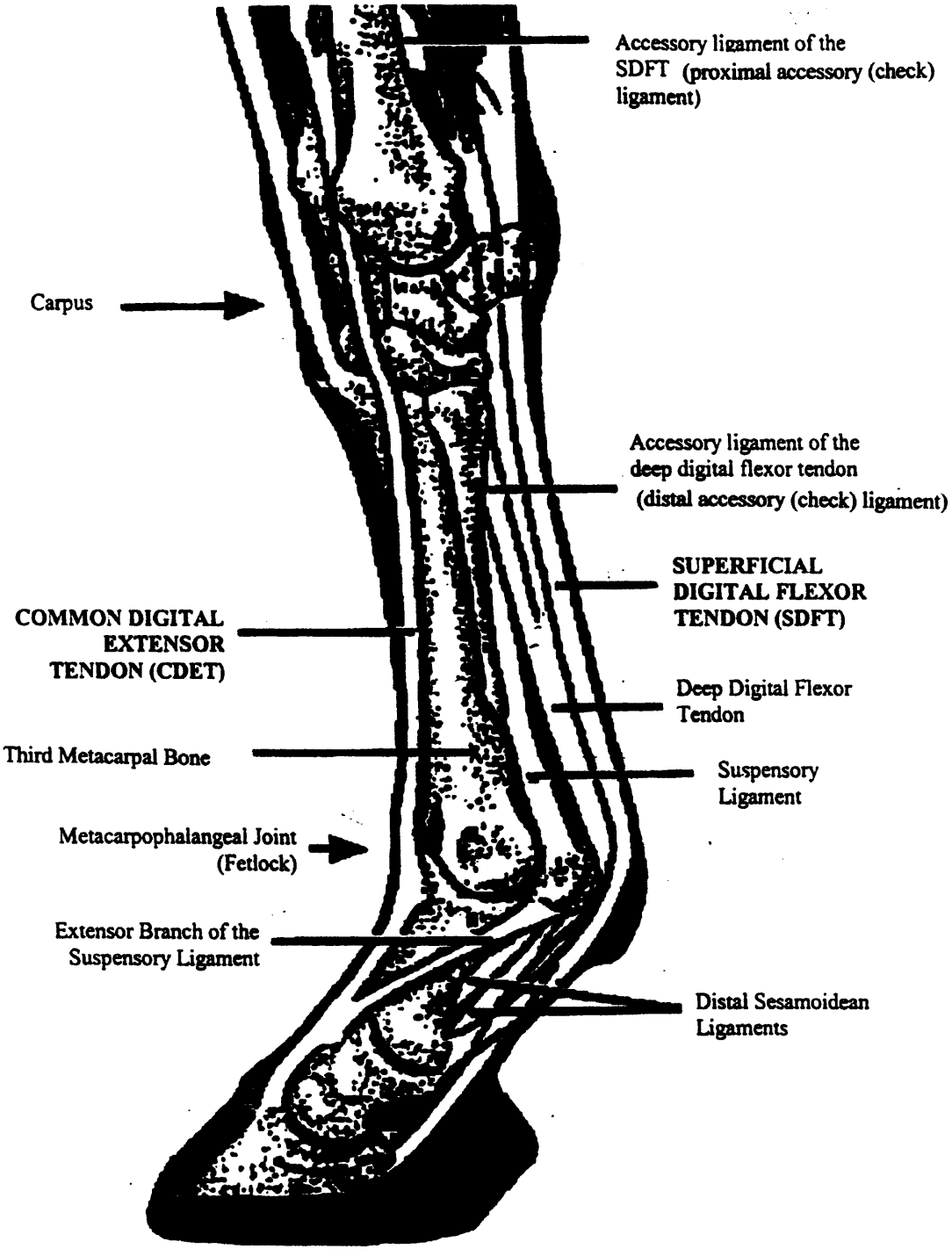


Figure 1.13: Diagrammatic representation of the anatomy of the distal equine forelimb.

Adapted from Kainer (1987).

Proximally, the SDFT and DDFT share a synovial sheath, which lubricates the tendons through the retinaculum, facilitating gliding movement of the tendons against each other. The sheath begins just proximally to the metacarpophalangeal joint and ends level with the middle of the middle phalanx. Distally, the DDFT is protected by the navicular bursa, which functions to protect the DDFT from excessive friction and pressure against the distal sesamoid bone.

1.9.1.2 Common and Lateral Digital Extensor Tendons

The CDET passes down the dorsal aspect of the metacarpal bone and over the dorsal pouch of the metacarpophalangeal joint capsule where it is protected by a synovial bursa. It broadens, before making some attachments at the proximal borders of the proximal phalanx and the middle phalanx before receiving the extensor branches of the interosseus to insert on the distal phalanx. The extensor tendon and the muscle, in conjunction with the action of the flexor tendons and their muscles, help to brace the horse's leg in such a manner that it may bear the weight of the horse (Parry, 1978b). The LDET runs down the dorso-lateral aspect of the metacarpal bone lateral to the CDET where it crosses the metacarpophalangeal joint and inserts on the dorsal aspect of the proximal phalanx. The LDET aids the CDET but does not have a particular role as due to evolution the horse no longer has a lateral digit so this tendon is a non-energy storing redundant tendon.

1.9.1.3 *Interosseous Muscle*

As mentioned earlier, the interosseous muscle is also known as the suspensory ligament and gets its name from its appearance as a strong, flat tendinous band, which plays a role in the support of the metacarpophalangeal joint. It arises from the palmar carpal ligament, descends distally down the metacarpal bone and divides into two branches just above the metacarpophalangeal joint. These branches insert on the proximal sesamoid bones and each branch winds around the proximal phalanx to join the CDET at the phalangeal joint. The suspensory ligament also functions as an elastic energy store.

1.9.2 The Human Achilles Tendon

As previously mentioned, the horse is an ideal model to study the effects of tendon injury in the human Achilles tendon, due to the fact that both the SDFT and Achilles tendon function as energy storing tendons and both sustain high rates of injury. The Achilles tendon is the strongest, thickest, and largest tendon in the human body (Sarrafian, 1983), and it plays an important role in stability of the musculoskeletal system. The Achilles tendon originates as a merger of the gastrocnemius and soleus muscles **(Figure 1.14).** During its distal descent, the fibres originating from each of these muscles rotate externally, creating a rope-like structure, so that, on insertion on the calcaneus, those fibres from the gastrocnemius are formed on the lateral aspect while those formed from the soleus are on the medial side (Cummings *et al.,* 1946). The narrowest part of the Achilles tendon is usually about 3 cm above its insertion onto the calcaneus. The Achilles tendon is under constant high stress (Ker *et al.,* 1988) as long as the individual remains standing. The Plantaris muscle has a short (10 cm) belly which ends in a long slender tendon called the Plantaris tendon, which descends between the gastrocnemius and soleus muscles, and then along the medial border of the Achilles tendon to insert into the calcaneus (Netter, 1987).

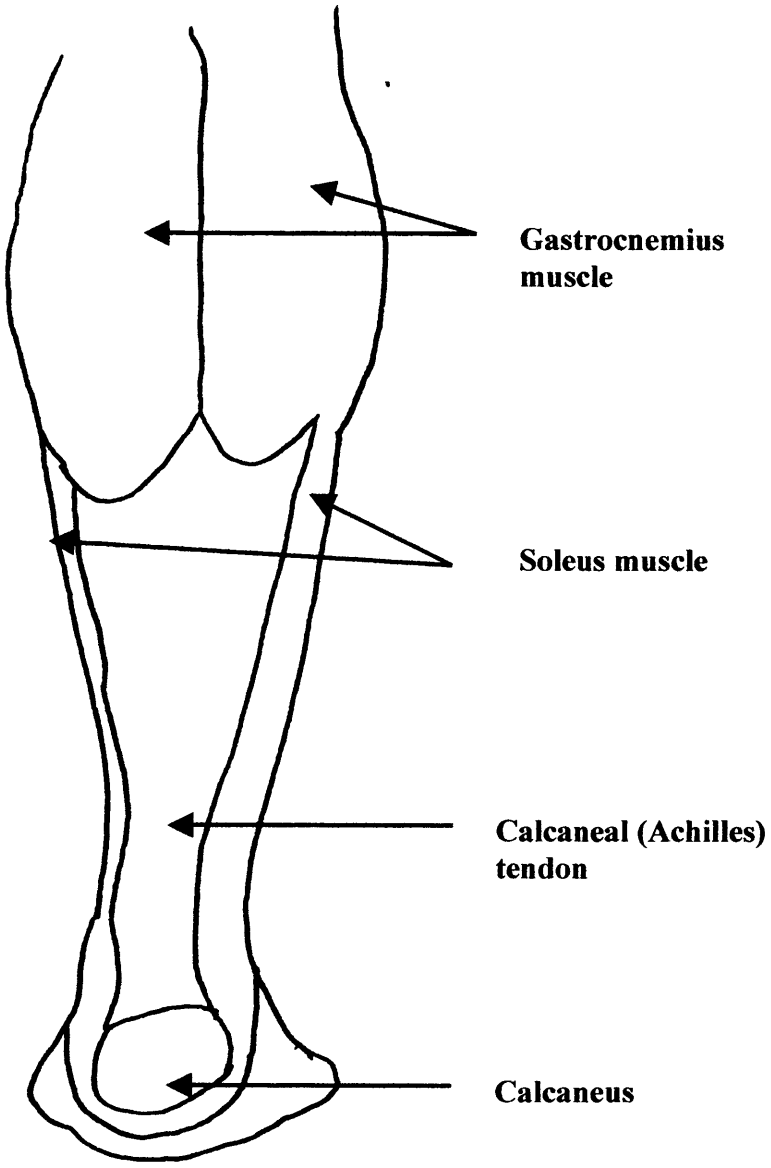


Figure 1.14: Human Achilles Tendon (Adapted from Netter, 1987).

CHAPTER 2

AIMS AND OBJECTIVES

2.1 AIMS

The aim of this thesis is to test a series of hypotheses developed under the overall goal of determining how the morphology of tendon is related to its structural and material properties. The aim of this thesis is to correlate matrix composition and morphological characteristics of tendons with their material and structural properties.

2.2 HYPOTHESIS

This study will explore the hypothesis that the mechanical properties of the SDFT can be predicted from an analysis of the matrix composition and morphology of the tissue.

2.3 OBJECTIVES

The main objectives of the study are:

1. To determine the structural and material properties, collagen fibril diameters and matrix composition of the SDFT and relate these to the function of this tendon (Chapter 4).

2. To determine structural and material properties, collagen fibril morphology, and matrix composition of energy storing structures (SDFT, SL) and non-energy storing structures (DDFT, CDET) from the equine distal forelimb. It will then be determined whether relationships exist between mechanical properties, collagen fibril morphology and matrix composition in tendons with different functions (Chapter 5).

3. To determine whether age related changes occur in mechanical properties, tendon fascicle, and fibril morphology in the SDFT. In particular, to determine whether tendon fascicle size and collagen fibril diameters decrease with age in the central core of the SDFT, which may account for site-specific tendon degeneration (Chapter 6).

4. To determine whether exercise levels affect mechanical properties, tendon fascicle, and fibril morphology in the SDFT. In particular to assess the influence of different horse types on tendon morphology and function (Chapter 6).

2.4 EXPERIMENTAL DESIGN

In the present study, the SDFTs of 30 horses were collected and structural and material properties determined on the SDFT from the left forelimb. Collagen fibril diameters, fascicle morphology, and matrix composition were determined on the right forelimb. Biochemical and ultra-structural properties were then compared to mechanical properties to determine whether the morphology and mechanical properties of the SDFT relate to the matrix composition (Chapter 4).

The SDFT, DDFT, CDET, SL of 6 of the 30 horses were collected and analysed for structural and material properties, collagen fibril diameters, and matrix composition to determine whether any relationships found in Chapter 4 are also found in tendons which have different functions i.e. energy storing versus positional tendons (Chapter 5).

Factors possibly influencing any relationships obtained in the present study are horse age and exercise level, which were further investigated in the SDFT group to determine whether age related changes occur in tendon mechanical properties, collagen fibril diameters, fascicle morphology, and matrix composition (Chapter 6).

2.5 STATISTICAL ANALYSIS

SPSS for Windows (12.0) was used for all analyses. Statistical significance was determined using Spearman's correlation to assess relationships between mechanical properties, morphology, matrix composition, and to assess changes with horse age. A general linear model was used to compare mechanical properties, morphology and matrix composition in different tendons. Statistical significance was taken as $p \leq 0.05$. Data are presented as mean ± standard deviation (SD).

CHAPTER 3

MATERIALS AND METHODS

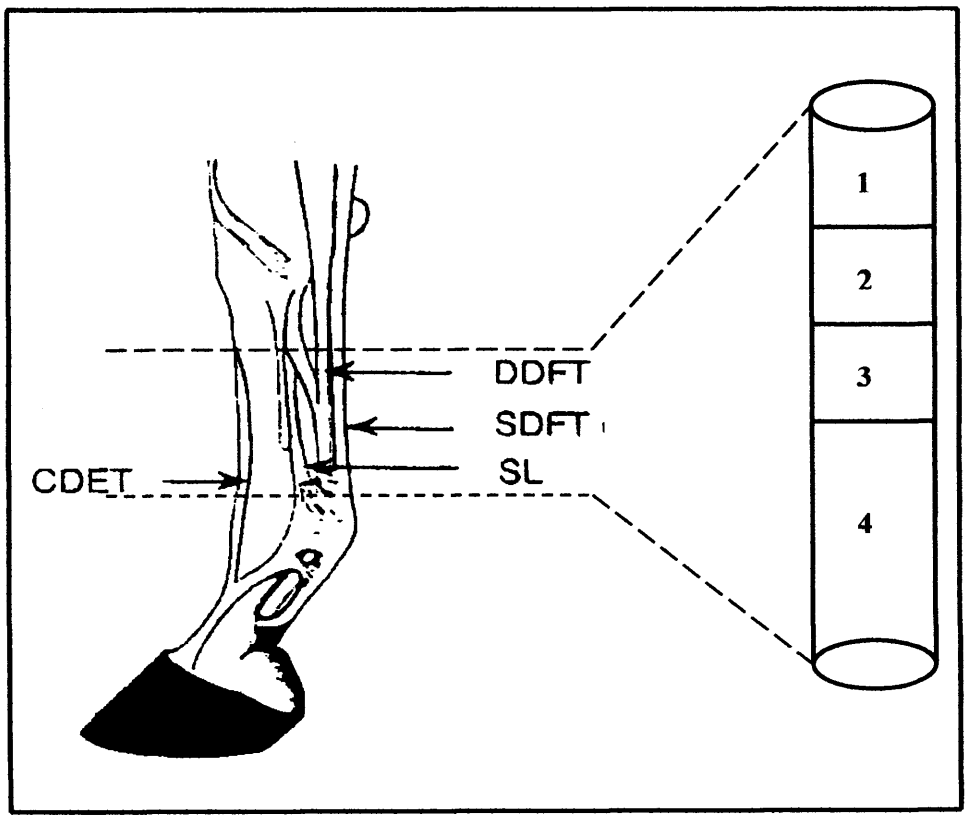
3.1 Tissue Collection

The whole forelimbs were collected from horses (n=30), ranging in age from 2-23 years (mean ± SD, 10.9 ± 6.1 yrs), destroyed at an abattoir for reasons other than tendon injury and used within 24 hours of death **(Table 3.1)**. SDFTs from both the left and right forelimbs were taken. From a subgroup of this main group of horses (n=6), ranging in age from 4-12 years (mean ± SD, 7.8 ± 3.1 yrs), the SDFT, DDFT, SL and CDET were taken from both the left and right forelimbs to investigate tendons with different functions **(Table 3.1)**. No significant differences in mechanical properties have previously been found between right and left forelimb SDFTs from the same horse (Birch *et al.*, 2000). The left tendon was tested mechanically to failure and the right tendon was used for histology, electron microscopy, and biochemistry. The right tendon was divided into four sampling sections within the mid-metacarpal region **(Figure 3.1)**.

The third metacarpal bone was measured from the distal carpal joint to the distal end of the third metacarpal bone at the metacarpophalangeal joint. This length was measured with a ruler and recorded, then the mid-metacarpal level was taken as half of this distance. The mid-metacarpal level (region most often injured) of the SDFT and CDET was then marked onto the tendon surface using a permanent marker pen (BDH Laboratory Supplies, UK). The DDFT was measured from the proximal point where the accessory ligament inserts into the tendon, to the widest distal part of the tendon. The SL was measured from the proximal point of origin to the distal region bifurcation. The most homogeneous part of the DDFT and SL in terms of size was then identified and this level was marked onto the tendon surface. The tendons were then dissected out from the level of the carpal joint to just below the metacarpophalangeal joint and wrapped in cling film (Tesco, UK) to reduce desiccation.

Table 3.1: Horse information database. (All of the 30 horses were used in Chapters 4 & 6. Left and right forelimb SDFTs were taken. * Represents the six horses used for the Chapter 5 study. Left and right forelimb SDFT, DDFT, SL and CDET tendons were taken).

Horse	Horse Type	Age	Exercise History
1	Dressage Horse (warmblood)	8	Dressage training
2	Racehorse (French Tb)	7	Race training
3	Racehorse (French Tb)	7	Race training
4	Thoroughbred	3	Race training, ill for a year and not trained
5	Irish Tb Ex-showjumper B Grade	16	Show jumped until 3 months ago
6	Thoroughbred	20	Racing, broodmare for 4 years
7	Thoroughbred	5	Not fast enough for racing
8*	Irish	7	Show horse
9	Thoroughbred Cross	9	Hunting and general riding
10*	Thoroughbred Cross	7	General purpose, hunt/dressage/jumping
11	Thoroughbred	15	Ex point to point, won lots of races
12	Thoroughbred	11	Racehorse, not raced for 2 years
13	Cob	5	Riding hack for lady
14	Hunter	23	Hunting
15*	Thoroughbred	4	History of hindlimb and forelimb ataxia
16*	Thoroughbred	11	Unknown
17*	7/8 Tb Irish Sport Horse/Eventing	6	Schooling
18	Thoroughbred	2	No training
19*	Thoroughbred type	12	Huntmasters horse
20	Thoroughbred	12	Cross-country, show jumping, hunting
21	Thoroughbred type	9	Intermediate event horse
22	Arab	7	Not much done
23	Thoroughbred type	20	Hunting/eventing
24	Unknown	20	General hack
25	Thoroughbred	13	Raced, bred 3 foals
26	Thoroughbred type	11	Unknown
27	Thoroughbred type	5	Unbroken
28	Polo pony	20	Polo retired
29	French warmblood	22	Hunting, retired 1 year ago
30	Thoroughbred type	9	Hunting



Section 1: 0.5 cm histology section taken for SEM (snap frozen whole).

Section 2: 0.5 cm histology transverse section taken (formalin fixed whole).

Section 3: 0.5 cm section for TEM (central and peripheral zone tissue 1 mm^2 x 5 mm longitudinal sections chopped out and fixed in gluteraldehyde).

Section 4: 2 cm section taken for molecular analysis (snap frozen whole, separated later into central and peripheral zone tissue).

Figure 3.1: Region of SDFT, DDFT, SL and CDET taken for analysis.

3.2 Cross-sectional Area Measurements

Loose connective tissue was removed from the tendon surface, and a mould was made of the tendon using alginate dental embedding paste (Blueprint Cremix, DENTSPLY DeTrey, Germany). The compound had set within 2 minutes of being placed into the mould. A slit was then cut along the mould and the specimen removed from the mould, and a transverse section was then cut at the mid-metacarpal level. A digital image was taken of the sectioned mould alongside a calibration scale **(Figure 3.2)**, using a 5.0 mega pixel digital camera with an 8 times optical zoom (Coolpix 5700, Nikon) mounted on a camera stand. Tendon cross-sectional area was measured using automated image analysis software (Image Pro Plus, Media Cybernetics UK). This technique was validated using metal rods of a known diameter and provides accurate values (within 0.8 %) for measuring CSA. This method was based on a technique developed by Goodship & Birch (2005).

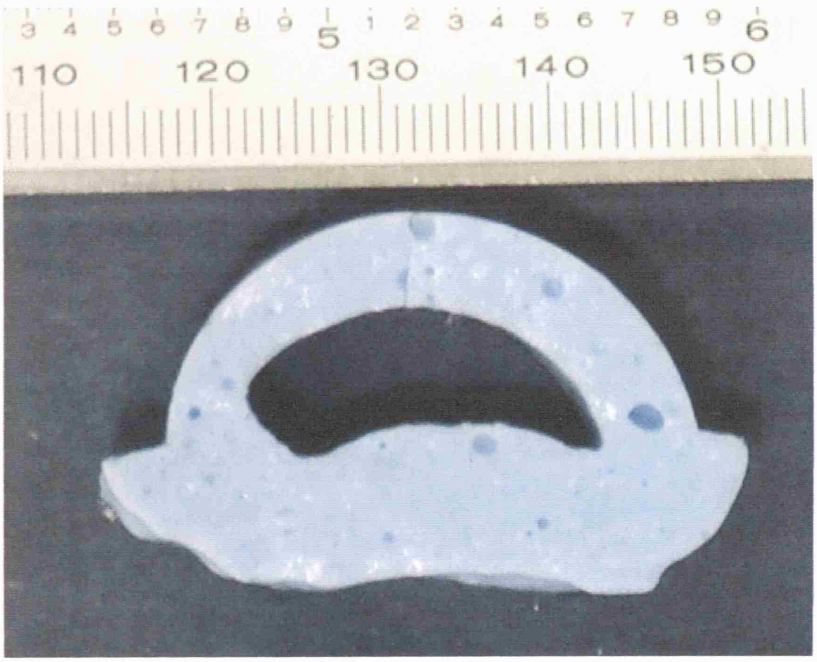


Figure 3.2: Cross-sectional area mould taken of SDFT using dental embedding paste and adjacent mm scale.

3.3 Mechanical Testing of Tendons

Tendons were tested in tension to failure in a servo-hydraulic materials testing machine (Dartec/Zwick, GmbH & Co., Ulm, Germany). The tendons were gripped using cryo-clamps (Riemersma and Schamhardt, 1982) to give a gauge distance between the clamps of 10 cm for the SDFT and CDET, and 8 cm for the DDFT and SL, with the level marked onto the tendon surface at dissection as the mid-point **(Figure 3.3)**. Clamps were lightly tightened before freezing to prevent slippage of the tendon, and then the tendon was frozen into the clamps using liquid CO_2. Clamps were then tightened to a torque of 27 Nm so that the tendon did not pull out during testing. A pre-load was applied to remove slack: 100 N for the SDFT, DDFT, SL and 25 N for the CDET, this being approximately 1% predicted load of failure. The gauge length of the tendon was measured and recorded from freeze line to freeze line prior to testing **(Figure 3.3)**. Twenty sinusoidal preconditioning cycles from pre-load to a load of 4.5 kN were applied to the SDFT, DDFT, SL and 1.5 kN to the CDET respectively, at a frequency of 1 Hz. The tendons were then loaded in tension to failure **(Figure 3.3)** at a strain rate of 80 % per second and a load versus deformation curve was plotted. Elastic modulus (force per unit area per unit extension in the linear region) of the material was calculated from the gradient of the linear region of the stress versus strain curve **(Figure 3.4)**. Ultimate tensile stress was recorded as the load at failure divided by the cross-sectional area of the tendon. Ultimate strain was defined as the percentage tendon deformation recorded at peak stress. Ultimate load was the maximum load at failure. 1 gram of tissue was taken after mechanical testing from the left forelimb SDFT, DDFT, CDET and SL at the level marked onto the tendon surface during dissection and this tissue was snap frozen and stored at –70 °C to be used for further molecular analysis.

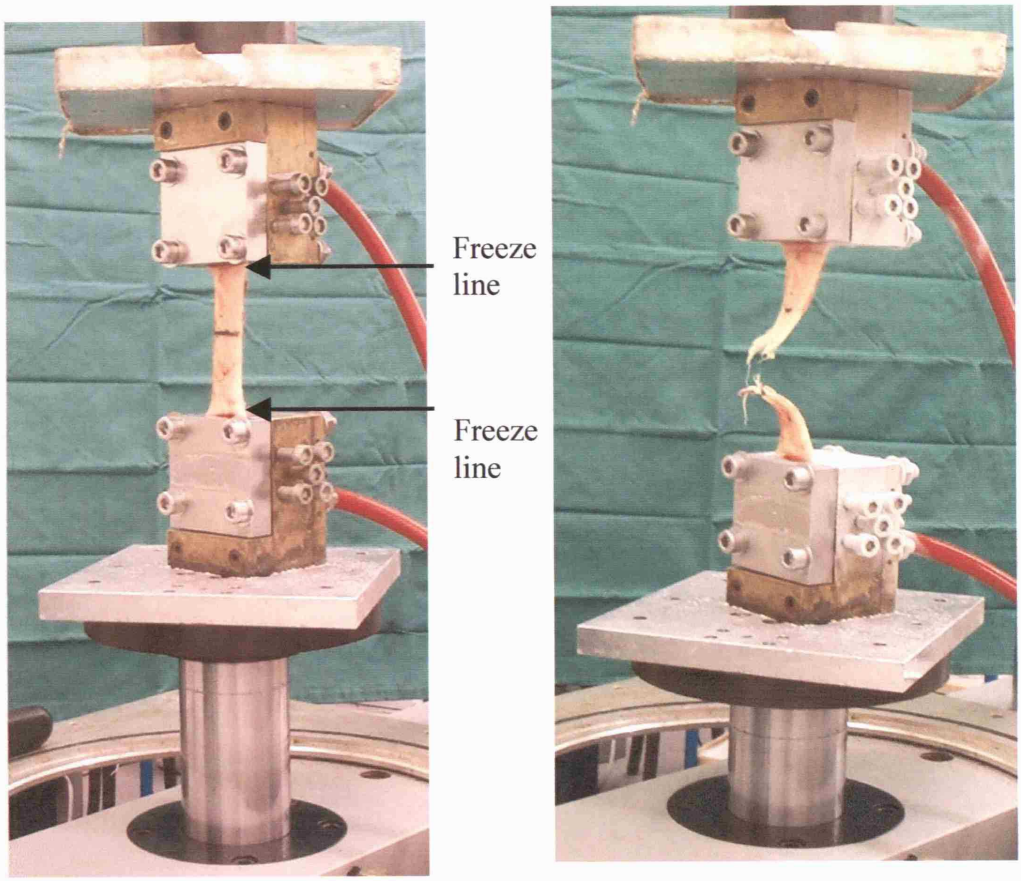


Figure 3.3: SDFT mounted in cryoclamps and tested to failure in a hydraulic materials testing machine.

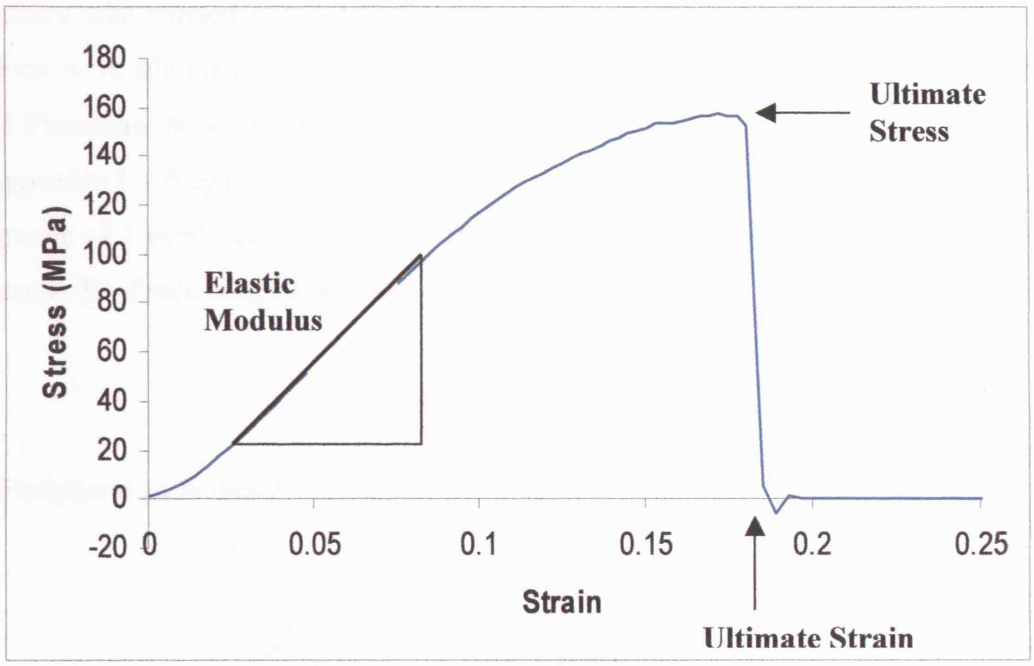


Figure 3.4: Example of a stress-strain curve of the SDFT following a 'break' test.

3.4 Collagen Fibril Diameters

Collagen fibril diameters were measured using a technique modified from that of Patterson-Kane *et al.* (1997b) using transmission electron microscopy (TEM). Modifications included samples being embedded in Spurr's resin (BDH Laboratory Supplies, UK) instead of Procure 812 resin (Probing & Structure, Australia), along with vacuum embedding, as this gave better infiltration and produced high quality electron micrographs suitable for measurement of collagen fibril diameters.

3.4.1 Tissue Processing

Five 1 mm^2 x 5 mm thick longitudinal samples from section 3 **(Figure 3.1)** were taken from central and peripheral zones of the SDFT and from the central zone of the DDFT, SL and CDET. From a subgroup of seven horse's, of which the SDFTs had been processed for TEM, both central and peripheral zone measurement of collagen fibril diameters was carried out to compare different zones for reproducibility **(Figure 3.5)**. From the remaining 23 horses, measurement of collagen fibril

diameters was carried out only on the central zone tissue of the SDFT. These sections were placed in 2 ml screw cap vials containing 2.5 % gluteraldehyde in 0.1M Phosphate buffer (BDH Laboratory Supplies, UK) for 3-4 days at 4 °C (Refer to Appendix I – Preparation of Chemicals). The samples were then cut into smaller fragments of 1 mm^3, left overnight in fixative and processed the next day (Refer to Appendix II - Processing Schedules).

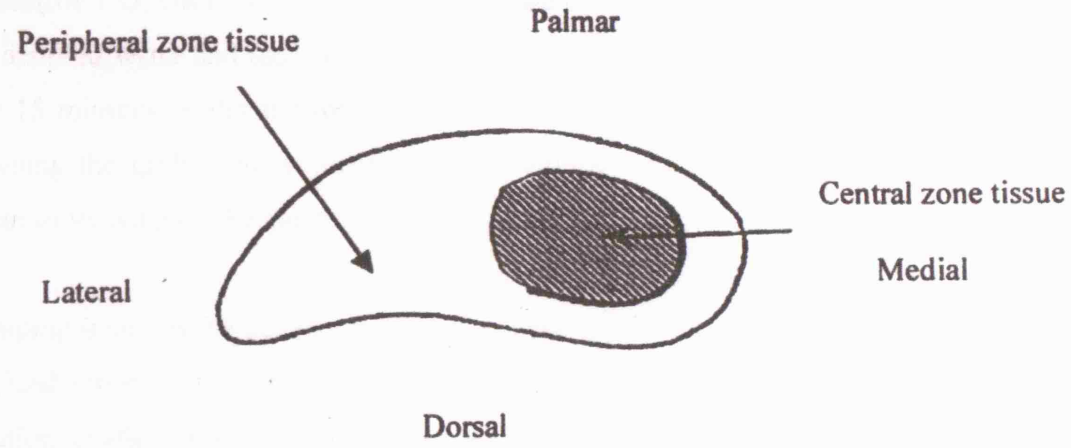


Figure 3.5: Diagrammatic representation of a transverse section through the SDFT at the mid-metacarpal level showing central and peripheral zone tissue.

3.4.2 Cutting Sections

Each block was mounted into the chuck of an ultramicrotome (LKB 8800A Ultratome III, Sweden), and the end trimmed into a pyramid shape using a double-edged razor blade **(Figure 3.6)**. The block surface was smoothed by cutting thick sections (1 µm) using a glass knife, which was prepared on a knife-making machine (LKB Knifemaker Type 7801B, Sweden). These thick sections were collected in a water trough made on the glass knife using wax to provide a seal for the water. They were transferred to a glass slide containing a drop of water, then heated onto the slide using a hot plate. The resulting sections were then stained with toluidine blue before viewing them under the light microscope to ensure correct transverse orientation of the sample.

Silver-gold sections (90-130 nm) were cut using a 3 mm diamond knife (Leica Microsystems UK Ltd, Milton Keynes), and at a cutting speed of approximately 2 mm/second. The sections were picked up from the water surface using 200 mesh formvar-coated copper grids (Agar Scientific Ltd, UK).

3.4.3 Staining

Grids were stained in drops of saturated uranyl acetate in 50 % ethanol (Agar Scientific Ltd, UK) for 15 minutes. Grids were then quickly dipped in three washes of distilled water and then submerged in drops of lead citrate stain (Reynolds, 1963) for 15 minutes (Refer to Appendix I – Preparation of Chemicals). Following lead staining the grids were washed again in distilled water and blotted on filter paper prior to viewing on the electron microscope.

Staining involves the deposition of heavy metal atoms onto the tissue through the use of lead citrate, which can scatter or stop electrons, increasing the contrast of the section (Patterson-Kane, 1996). Uranyl acetate increases the contrast of tissue proteins such as collagen when used prior to staining with lead (Griffin, 1990).

3.4.4 Electron Microscopy

The sections were viewed using a Philips CM12 analytical transmission electron microscope (FEI, Cambridge, UK). One representative section was viewed for each tendon and the areas of each section from which micrographs were taken were selected randomly. Ten photos were taken per section, which was sufficient to allow analysis of approximately 1000 collagen fibril diameters. All sections were photographed at a magnification of 45000 times and a carbon grating replica was used to calibrate the microscope.

Micrographs were recorded on Kodak electron microscope film and developed in a high contrast developer (Kodak, D19, UK). The prints were enlarged by a factor of 1.4 using an enlarger (Devene Ltd, Kent, UK), developed onto photographic paper (Kentmere Photographic Ltd, Cumbria, UK), and then fed through a print drier (RCD 3200).

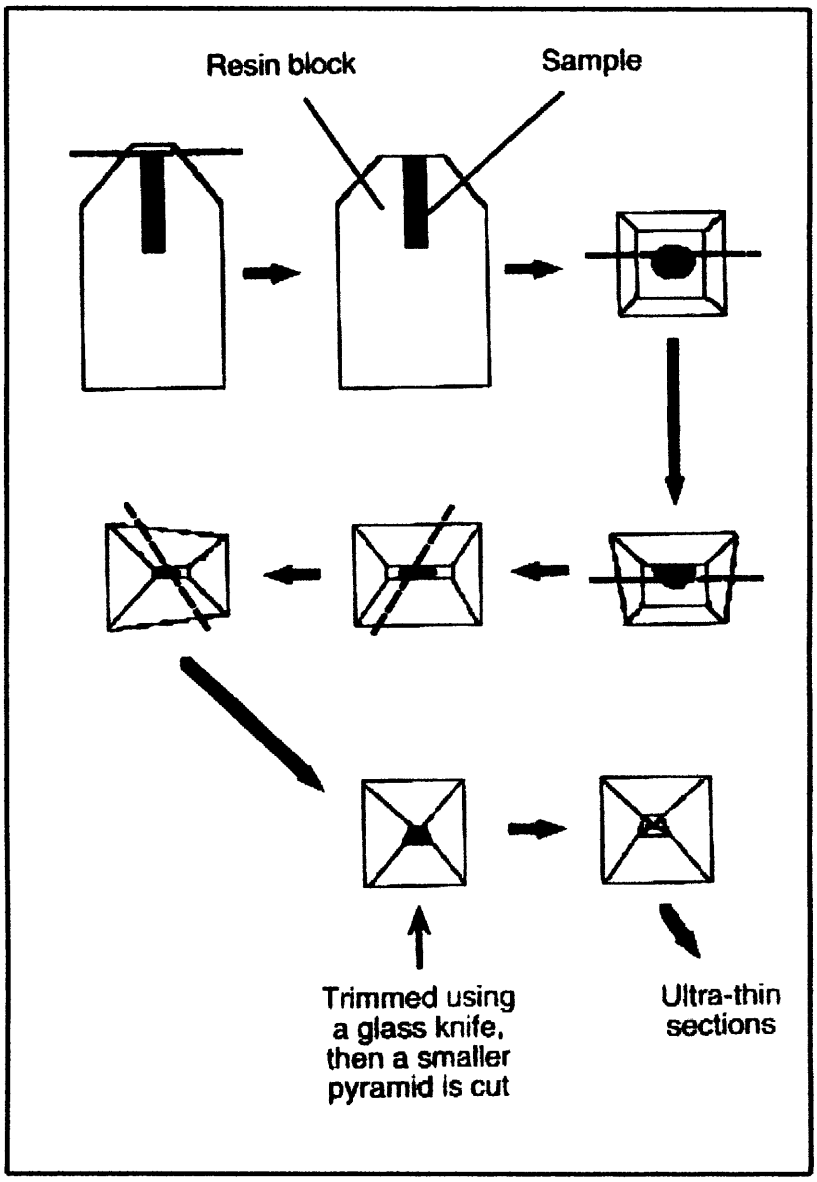


Figure 3.6: TEM sectioning diagram (Adapted from Griffin, 1990).

3.4.5 Computerised Image Analysis of Electron Micrographs

Micrographs were scanned alongside a calibration scale (Hewlett Packard flatbed scanner) to obtain a digital image and analysed using image analysis software (Image Pro Plus, Media Cybernetics UK). It has been calculated that measurement of at least 1000 collagen fibril diameters is sufficient to obtain a representative collagen fibril diameter distribution at a given site (Parry & Craig, 1977). Therefore,

approximately 1000 collagen fibril diameters were measured over a representative number of micrographs, and the measurements were imported into Microsoft Excel where a macro was run on the data to calculate the mass average collagen fibril diameter (MAD). This macro uses a calibration factor to convert the collagen fibril diameter measurements into nanometres and then calculates the percentage of total measured fibril area occupied by each diameter group.

The morphology of a fibril population may be characterised by the MAD. If each diameter value is plotted against the percentage of total collagen fibril area that is occupied by fibrils of that diameter, the MAD is the mean of this distribution. The MAD therefore differs from the simple mean diameter in that it takes into account the fact that the usually small numbers of large diameter fibrils can occupy a large proportion of the cross-sectional area of a sample (Patterson-Kane *et al.*, 1997a). The collagen fibril diameter distribution, which is the % total number of fibrils versus the fibril diameter, was also measured. Collagen fibrils can either have a unimodal distribution whereby fibrils are all of the same size, or they can have a bimodal distribution whereby fibrils are of two different sizes, i.e. large and small diameter fibrils. In addition to measuring the MAD, the collagen fibril index (CFI) was measured for a subgroup of 10 of the SDFTs. The CFI is the percentage of area covered by collagen therefore is a measure of the collagen to noncollagen ratio in the extracellular matrix (Enwemeka *et al.* 1992). The total area of each image was recorded and from this the percentage of each image area occupied by collagen fibrils was calculated to determine the CFI. Although indicative of relative collagen density, this variable does not provide information about the diameter of collagen fibrils (Cherdchutham *et al.* 2001).

3.5 Fascicle Morphology

Tendon morphology was investigated through the use of transverse sections of resin-embedded equine tendons based on the method of Gillis *et al.* (1997).

5 mm transverse sections from section 2 **(Figure 3.1)** were fixed in 10 % neutral buffered formalin (BDH Laboratory Supplies, UK) for 1 week. The sections were all processed following the conventional method of Bancroft & Stevens (1990) and

embedded using L.R. White Resin (Agar Scientific Ltd, UK), a hard grade acrylic resin (Refer to Appendix II – Processing Schedules).

3.5.1 Embedding Samples

Samples were embedded using peel-away disposable plastic moulds (Science Services, London, UK) and the "cold" curing method. The base of each plastic mould was smeared with a drop of accelerator. A further 1 drop of accelerator was mixed with every 10 ml of resin used, and this was poured into the mould once the tissue was orientated correctly. Polymerisation occurred within 10-20 minutes. Samples were left to polymerise on the bench and once the moulds began to heat up they were transferred to the –20 °C freezer until they had completely cooled down. Samples required cooling with ice to prevent cracking due to the exothermic reaction occurring during polymerisation.

3.5.2 Mounting Resin Sections

Following polymerisation, the blocks were removed from the moulds. The top surface of the block was ground flat with p240 sandpaper and mounted onto a plastic slide with acrylic glue. The bottom surface of the block was then ground and polished on a Buehler Motopol 2000 Grinder and Polisher, using p240 sandpaper, until the tendon surface was visible and surface scratches had been smoothed out. This surface was then mounted onto another plastic slide using Technovit (EXAKT, Germany) and placed into a clamping apparatus **(Figure 3.7)** to ensure adequate fixation. Technovit requires UV light for fixation so the clamping apparatus was placed under UV light for 1 hour in the fumehood with protective covers placed in front of it. The blocks were then taken out of the clamping apparatus and placed on the UV lamp for a further 30 minutes.

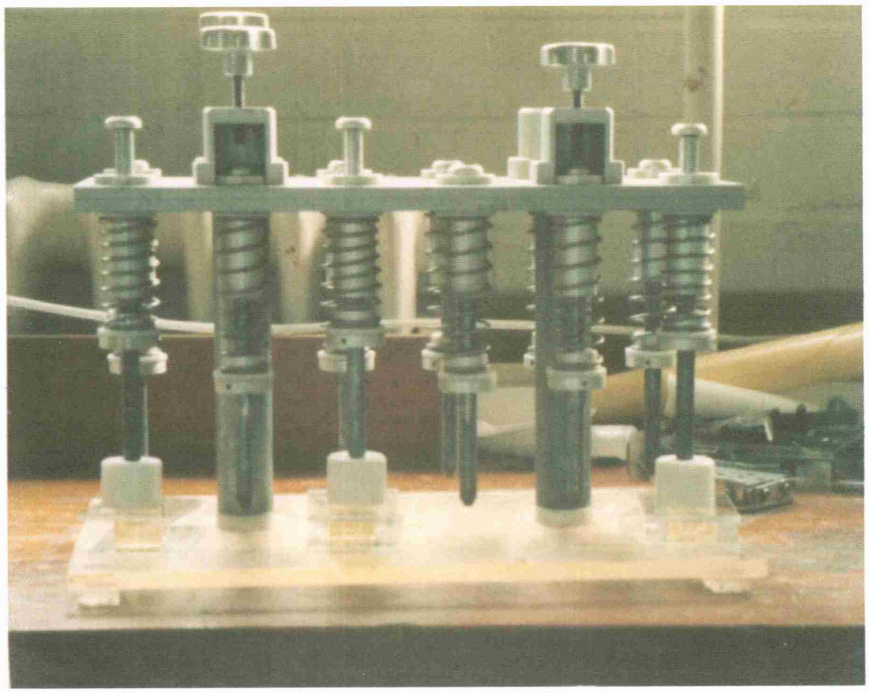


Figure 3.7: Clamping apparatus used in resin histology.

3.5.3 Cutting Resin Sections

Sections 1 mm thick were cut using the Buehler Isomet 2000 motorised saw, complete with a 0.2 mm diamond blade. These sections were then ground down to between 150 and 200 μm using p240 and lower grade sandpaper. They were then fine polished using alumina polish to remove surface scratches, which were observable under the light microscope.

3.5.4 Staining

The slides were stained using the Paragon Method (Refer to Appendix I – Preparation of Chemicals). Slides were placed in toluidine blue for 10 minutes, then rinsed thoroughly and blotted dry. They were then stained for 15 minutes in Paragon Multiple Stain, rinsed thoroughly, dehydrated and mounted with DPX (BDH Laboratory Supplies, UK). The slides were then ready for viewing under the light microscope. Paragon stained the cell nuclei blue and soft tissue components of the

tendon stained a pink to purple colour. This allowed the presence of tenocytes, blood vessels, interfascicular septa and fascicles to be identified.

3.6 Matrix Composition

A 2 cm section was taken for biochemical analysis from section 4 of the right forelimb SDFT, DDFT, SL and CDET **(Figure 3.1)**. This tissue was snap frozen whole and separated later into central and peripheral region tissue **(Figure 3.5)** prior to biochemical analysis.

3.6.1 Water Content

Tendon sections were thawed at room temperature and the loose connective tissue and paratenon removed. Water content was determined by weighing out approximately 1 gram of tissue from central and peripheral regions of the SDFT, along with 1 gram of tissue from central regions of the DDFT, SL and CDET. Tissue was frozen at –70 °C and lyophilised in a freeze drier overnight until a constant weight was reached. Lyophilised tissue was then reweighed to give the dry weight. Water content was expressed as a percentage of the wet weight.

3.6.2 Collagen Content

Freeze dried tissue was ground up in a mechanical grinder (Retsch, MM2000) and approximately 20 mg weighed out accurately. Tissue was digested with papain for 24 hours at 60 °C which completely solubilised the tissue (Kim *et al.* 1988). Collagen content was determined by measuring the imino acid hydroxyproline. An aliquot of papain digest (100 µl) was hydrolysed in 6 M/l HCL at 110 °C for 24 hours, dried under a vacuum and the residue dissolved in de-ionised water. Hydroxyproline was assayed using Ehrlich's reagent by a method developed from that of Bergman & Loxley (1963). Hydroxyproline concentrations were calculated by comparison with a standard curve prepared with hydroxyproline standards (1-10 µg hydroxyproline/ml) and collagen content calculated assuming hydroxyproline to be present at 14 % (Birch *et al.* 1998). Collagen content was expressed as a percentage of the dry weight of the tendon tissue.

3.6.3 Glycosaminoglycan Assay

A total sulphated glycosaminoglycan (GAG) assay was carried out on papain-digested tissue using the dimethylmethylene blue dye method of Farndale *et al.* (1986). Total sulphated GAGs were calculated by comparison with a standard curve prepared with purified bovine trachea chondroitin sulphate (0-10 μg in 3 ml dye). Results were expressed as μg chondroitin sulphate equivalent sulphated GAG/mg dry weight tissue.

3.6.4 Deoxyribonucleic Acid (DNA) Assay

Lyophilised tissue (10 mg) was suspended in 2 ml sterile PBS (phosphate buffered saline), pH 6.0, plus 5 mmol/l cysteine.HCL and 5 mmol/l EDTA (ethylenediamine tetra-acetic acid). Papain was added at (125 μg/ml) and digestion carried out at 60 °C for 24 h to completely solubilise the tissue. DNA was assayed in the papain digests by the fluorometric method of Kim *et al.* (1988) using the bisbenzimidazole dye (Hoechst 33258). DNA concentrations were calculated by comparison with a standard curve prepared with calf thymus DNA (1-10 μg DNA/ml sterile PBS) and diluted in dye solution to give a range of concentrations from 0.5 to 12.5 μg/ml. DNA content is expressed as μg DNA/mg dry weight tissue.

3.7 Scanning Electron Microscopy (SEM)

SEM was used to investigate tendon transversely and to quantitatively measure fascicles in the central core (site of degeneration), medial peripheral (MP) and lateral peripheral (LP) zones of the SDFT **(Figure 3.8)**.

A 5 mm transverse section from each SDFT was snap frozen and stored at –70 °C. Each sample was then lyophilised in a freeze drier overnight, mounted onto an aluminium stub, coated with gold paladium and viewed under the SEM (Jeol JMS 5500LV). The cross-sectional area (CSA) and septal width of 10 fascicles from the central core, medial peripheral (MP), and lateral peripheral (LP) zones of the SDFT were measured using image analysis software (Image Pro Plus, Media Cybernetics UK). An additional 5 mm transverse section just above section 1 **(Figure 3.1)** was

taken and lyophilised in a freeze drier overnight without being snap frozen, mounted onto an aluminium stub, coated with gold paladium and viewed under the SEM in the same manner as above.

Two photos were taken per zone on the SEM at 33 X magnification for each SDFT in order to obtain the measurements from 10 fascicles. Only fascicles from which the boundaries could be determined were used in this study. The irregular shape of fascicles can make this difficult. Fascicles from MP and LP zones of the SDFT, which were greater than 2 mm from the edge of the tendon were classified as central core fascicles, so were measured in the central core category.

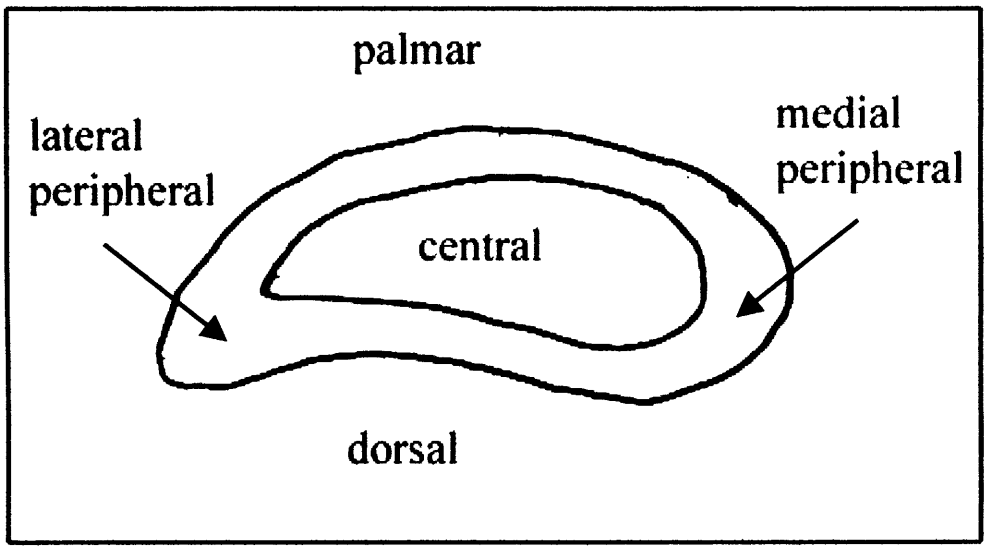


Figure 3.8: Diagrammatic representation of medial peripheral (MP), central and lateral peripheral (LP) sampling zones in the SDFT.

3.8 Statistical Analysis

Statistical significance was determined using Spearman's correlation to assess the relationship between mechanical properties, collagen fibril morphology, and matrix composition in the SDFT (Chapter 4). This was a nonparametric measure between two ordinal variables. A bivariate correlation was used to measure how variables were related and determine evidence of a linear relationship.

Statistical significance was determined using a general linear model (mixed model analysis) to compare material properties, collagen fibrils and matrix composition in the SDFT, DDFT, SL and CDET (Chapter 5). This was a univariate analysis of variance for the effect of tendon (fixed factor) and horse (random factor) on the different outcomes. Spearman's correlation was used to assess the relationship between elastic modulus, collagen fibril diameters, water content, collagen content, and GAG content in the different tendons.

Statistical significance was determined using a general linear model to compare fascicles and fibrils from different zones of the SDFT and Spearman's correlation to assess changes with horse age and exercise effects (Chapter 6). Statistical significance was taken as $p \leq 0.05$. Data are presented as mean ± SD.

CHAPTER 4

HOW DOES COLLAGEN FIBRIL MORPHOLOGY RELATE TO MATRIX COMPOSITION AND MECHANICAL PROPERTIES OF THE SDFT?

4.1 INTRODUCTION

4.1.1 Mechanical properties of the SDFT

The optimisation of mechanical properties such as strength and stiffness is essential for the SDFT to function efficiently. The SDFT needs to be highly elastic, i.e. have a low stiffness, in order to stretch and return a maximum amount of stored energy. It is known that the SDFT has a range of strength and stiffness (Birch *et al.*, 2000). This is important because this tendon is most often injured. It has previously been shown that within a population of horses, the mechanical properties of the SDFT vary considerably (Batson *et al.* 2003; Birch *et al.* 2000; Gillis *et al.* 1995; Wilson & Goodship, 1990) suggesting that those horses with weaker tendons may be those most prone to tendon injury. Through the ability to identify such horses, it may be possible to predict which horses are more at risk.

Several studies have investigated the mechanical properties of the SDFT (Abrahams, 1967; Herrick *et al.* 1978; Jansen & Savelberg, 1994; Lochner *et al.* 1980; Riemersma & Schamhardt, 1985; Stephens *et al.* 1989) either *in vitro* or *in vivo*. However, the results obtained for stress and strain values are rather disparate and often contradictory, likely because of differences in experimental protocol. High tendon stresses (>50 MPa) occur in a variety of mammals including the horse (Jansen & Savelberg, 1994), dog, deer and human (Ker *et al.*, 1988). Herrick *et al.* (1978) tested normal SDFT *in vitro*, using cyclic compression, and observed that strains up to 12 % can be sustained without causing damage to the tendons. In another study performed *in vivo* and *in vitro*, Lochner *et al.* (1980) suggested that 'at the walk', strain of up to 25 % could be present in the SDFT of the horse. However, more recently, results have been in agreement that the SDFT ruptures *in vitro* at between 12-20 % strain, while *in vivo* measurements have demonstrated strains of up to 16 % at the gallop (Goodship *et al.* 1994; Riemersma & Schamhardt, 1985; Stephens *et al.* 1989, Wilson & Goodship, 1990). Thus at peak locomotor performance, the SDFT is operating close to the level of tensile failure and it can be seen how narrow the safety margin is for elastic energy storage in this structure.

Previous studies into the mechanical properties of tendons have shown that different tendons have specific structural, material, and matrix properties, which may relate to physiological function. Batson *et al.* (2003) demonstrated that there are significant differences in material and structural properties, and matrix molecular composition between the SDFT and CDET, although this study did not include an investigation of morphology in terms of collagen fibril diameters. Crevier *et al.* (1996) studied the mechanical behaviour of different regions of the equine SDFT and found no significant difference in the elastic modulus, between different segments of the tendon. Riemersma & Schamhardt (1985) studied *in vitro* mechanical properties of equine hindlimb tendons in relation to cross-sectional area and collagen content. They found that the modulus of elasticity varies along the SDFT and that the collagen content was inversely proportional to the CSA and proportional to the modulus of elasticity. However, these studies have been limited to measuring only one or two variables at the structural/mechanical/molecular level. The lack of integrative studies makes it difficult to investigate causal interactions in the mechanical properties of tendon with matrix composition and structure. It is not presently known whether a causal relationship exists within a specific tendon between mechanical properties, matrix composition, and collagen fibril morphology, as this has not been carried out in a single study. Therefore, this will be the main focus of the present study.

4.1.2 Significance of biochemical and ultra-structural properties

The mechanical properties of the SDFT are a consequence of both the extracellular matrix components and also their structural organisation. The collagen fibrils are the major units of tensile strength of the tendon (Parry *et al.*, 1978a). It has been postulated that fibril diameter distribution is associated with, and may be determined by the specific types of glycosaminoglycan (GAG) present, which alter with functional requirements (Parry *et al.*, 1982). The GAGs being negatively charged macromolecules, are also important in determining the matrix water content by exerting a strong swelling pressure (Scott, 1995). The total amount of collagen present will affect tendon mechanical properties as well as the way in which collagen molecules are organised and packed together. It has been suggested that large diameter collagen fibrils have a greater tensile strength than an equivalent mass of

collagen arranged in small fibrils, whereas small diameter collagen fibrils increase the ability of the tissue to withstand creep (Parry *et al.* 1978b). A positive correlation has previously been shown between the mass average collagen fibril diameter (MAD) and the ultimate strength of tendon, skin, and cartilage (Bailey *et al.*, 1970a,b; Parry & Craig, 1988). Therefore, collagen fibril diameters may also be expected to correlate with material properties of tendon tissue.

4.2 Hypothesis

The elastic modulus of the SDFT can be predicted from an analysis of the collagen fibril diameters, total sulphated glycosaminoglycans (GAGs), and water content components of matrix composition of the tissue.

4.3 Objectives

1. To determine structural and material properties, and to measure collagen fibril diameters, and matrix composition of the SDFT.

2. To compare the structural and material properties, collagen fibril diameters, and matrix composition in the SDFT and relate these to the function of this tendon.

4.4 Experimental Design

In this part of the study the mid-metacarpal region of the SDFT was chosen to carry out an investigation of matrix composition and collagen fibril morphology because injury is far more common in this region (Smith, 1894). The left forelimb SDFT was mechanically tested to determine the following structural and material properties: tendon cross-sectional area, ultimate load, % ultimate strain, structural stiffness, elastic modulus, and ultimate stress. The right forelimb SDFT was used to investigate the organisation of collagen in the matrix and this was assessed by measuring collagen fibril diameters and the following matrix composition: water content, collagen content, glycosaminoglycan content, and DNA content. From this, it will then be possible to relate biochemical and ultra-structural findings to the

material properties from the SDFT of the contralateral limb, to determine whether the morphology and material properties of the SDFT relate to the matrix composition.

4.5 RESULTS

4.5.1 Structural Properties

Structural properties varied widely between SDFTs **(Table 4.1)**. Strength and stiffness can be defined by measuring the tendon as a whole structure (structural stiffness and ultimate load), which does not take into account the size of the tendon. Tendon ultimate strength values ranged from 8-18 kN **(Figure 4.1)**, suggesting that some tendons are more than twice as strong as others. Ultimate strength values showed a normal distribution. Percentage ultimate strain values ranged from 8-20 % in the SDFT. Tendon structural stiffness values ranged from 0.84 – 1.62 N/mm, indicating that some tendons were up to twice the structural stiffness of others.

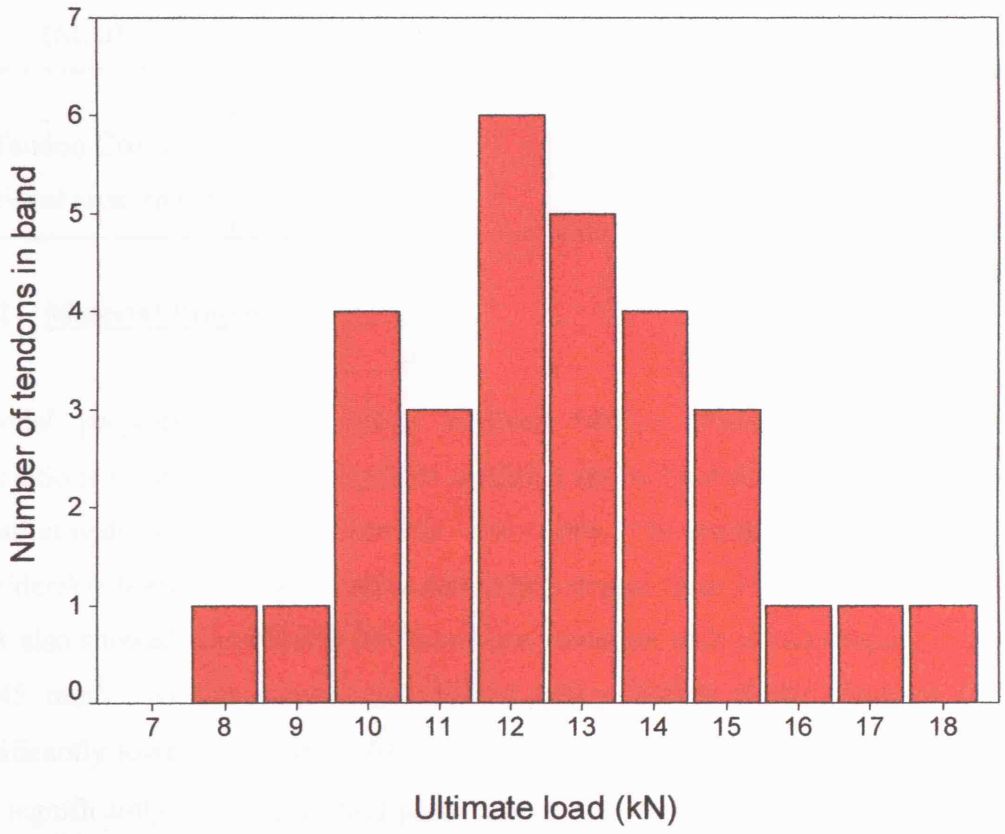


Figure 4.1: Distribution of tendon ultimate force values for the SDFT.

Table 4.1: Structural and Material Properties of the SDFT.

	N	MINIMUM	MAXIMUM	MEAN	STD DEVIATION
Ultimate Load (kN)	30	8	18	13	2
Ultimate Strain (%)	30	8	20	15	3
Structural Stiffness (N/mm)	30	0.84	1.62	1.15	0.18
Elastic Modulus (MPa)	30	902	1614	1197	180
Ultimate Stress (MPa)	30	91	187	132	25
Tendon Cross-sectional area (mm^2)	30	64	145	98	21

4.5.2 Material Properties

Material properties varied widely between SDFTs **(Table 4.1)** and several correlations were evident. The elastic modulus showed considerable horse to horse variation with values ranging from 902 - 1614 MPa. The ultimate stress also showed considerable horse to horse variation and values ranged from 91 – 187 MPa. Tendon CSA also showed considerable horse to horse variation with values ranging from 64 – 145 mm^2. Results showed that horses with a higher elastic modulus had a significantly lower ($n=30$, $p \leq 0.001$) tendon CSA **(Figure 4.2)**. The elastic modulus was significantly ($n=30$, $p \leq 0.001$) positively correlated with ultimate stress **(Figure 4.3)**. Therefore, tendons with a higher ultimate stress also had a significantly lower ($n=30$, $p \leq 0.001$) tendon CSA.

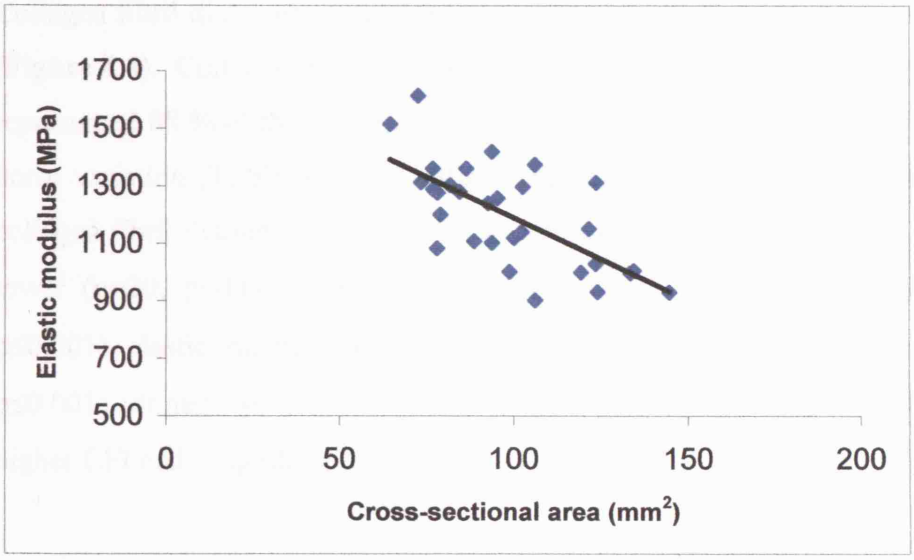


Figure 4.2: Elastic modulus values in the SDFT against tendon CSA (R^2=0.4568, n=30, p≤0.001).

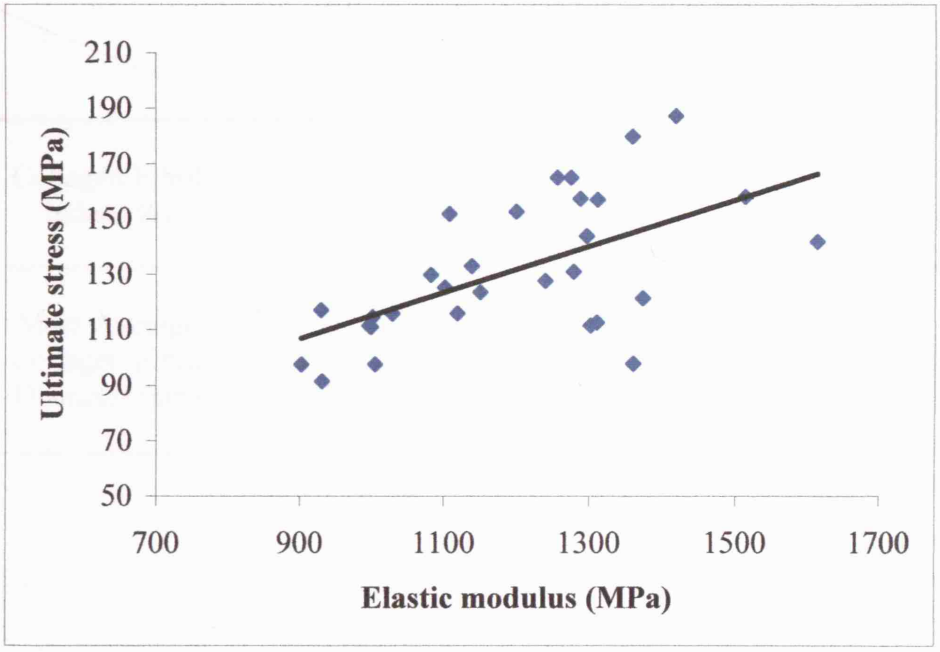


Figure 4.3: Relationship between the elastic modulus and ultimate stress of the SDFT (R^2=0.3484, n=30, p≤0.001).

4.5.3 Collagen Fibril Diameters

Collagen fibril diameters ranged from 20-380 nm and showed a bimodal distribution **(Figure 4.4)**. Collagen fibrils, in terms of numbers, with a diameter less than 160 nm represented 88 % of the total in the SDFT. The MAD showed considerable horse to horse variation **(Table 4.2)** and values ranged from 125 – 229 nm. Results of the collagen fibril diameters showed that horses with a higher MAD had a significantly lower ($n=30$, $p \leq 0.001$) tendon CSA **(Figure 4.5)**, a significantly higher ($n=30$, $p \leq 0.001$) elastic modulus **(Figure 4.6)**, and also a significantly higher ($n=30$, $p \leq 0.001$) ultimate stress. CFI values ranged from 53.3 – 67.8 %. Horses with a higher CFI had a significantly higher ($n=10$, $p=0.046$) MAD **(Figure 4.7)**.

Table 4.2: Morphological Composition of the SDFT.

	N	MINIMUM	MAXIMUM	MEAN	STD DEVIATION
Collagen Fibril Index (%)	10	53.3	67.8	61.2	4.3
Mass Average Collagen Fibril Diameter (nm)	30	125	229	169	25

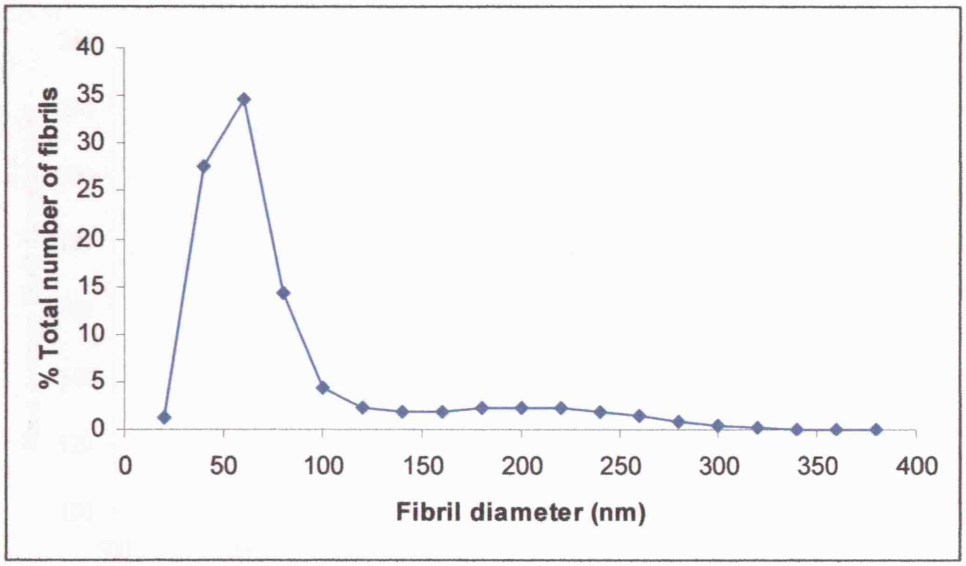


Figure 4.4: Collagen fibril diameter distribution of the SDFT.

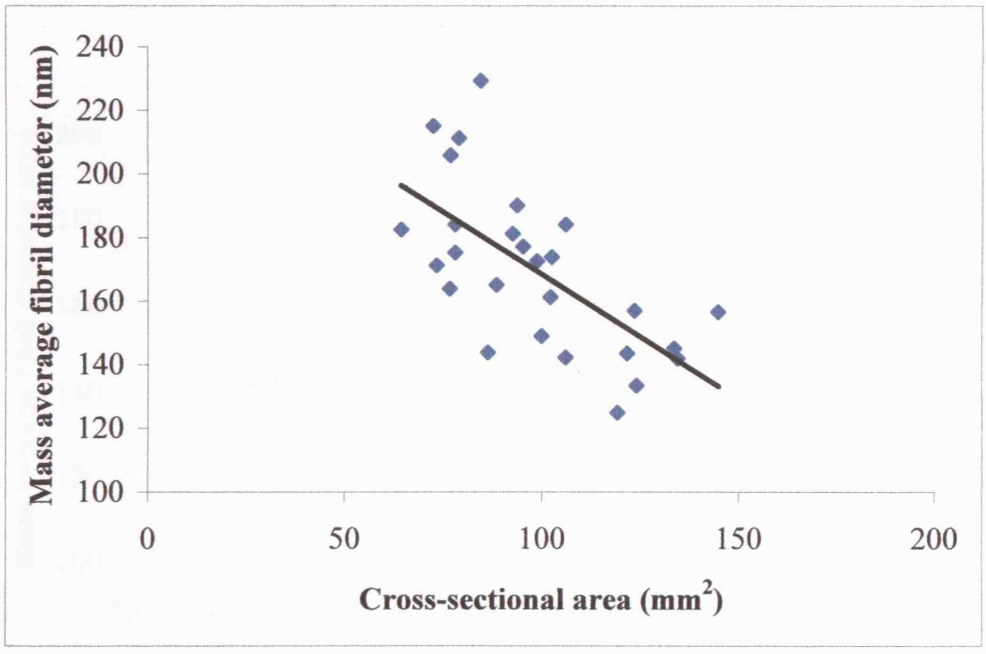


Figure 4.5: Mass average collagen fibril diameter in the SDFT against tendon CSA (R^2=0.4292, n=30, p≤0.001).

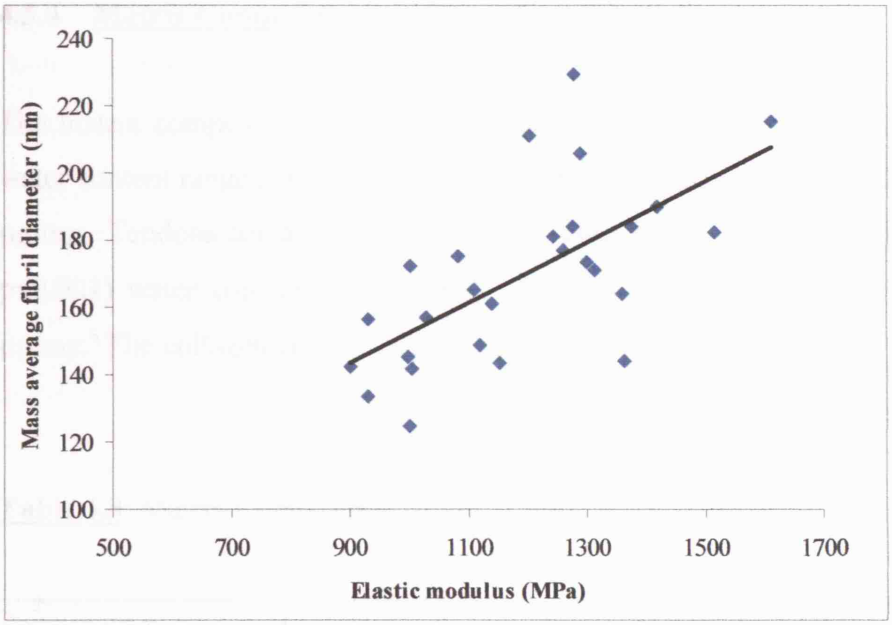


Figure 4.6: Mass average collagen fibril diameter in the SDFT against elastic modulus (R^2=0.4129, n=30, p≤0.001).

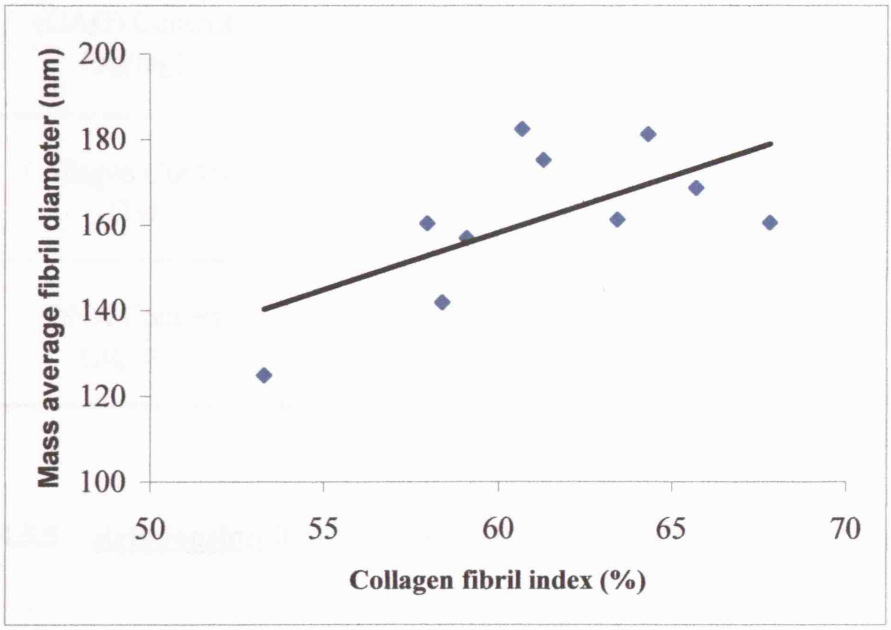


Figure 4.7: Mass average collagen fibril diameter (MAD) in the SDFT against collagen fibril index (CFI). (R^2=0.4108, n=10, p=0.046).

4.5.4 Matrix Composition

The matrix composition of the SDFT is represented in **Table 4.3**. The percentage water content ranged from 60.2 – 67.5 %. The GAG content ranged from 2.9 – 18.5 μg/mg. Tendons containing a higher GAG content had a significantly higher (n=30, $p \leq 0.001$) water content **(Figure 4.8)**. The DNA content ranged from 0.3 – 0.77 μg/mg. The collagen content ranged from 75 – 92 %.

Table 4.3: Matrix Composition of the SDFT.

	N	MINIMUM	MAXIMUM	MEAN	STD DEVIATION
Water Content (%)	30	60.2	67.5	64.7	1.7
Total Sulphated Glycosaminoglycan (GAG) Content (μg/mg)	30	2.9	18.5	8.2	2.9
Collagen Content (%)	30	75	92	85	4.7
DNA Content (μg/mg)	13	0.3	0.77	0.57	0.13

4.5.5 Relationship Between Mechanical Properties and Matrix Composition

The results showed that SDFTs containing higher water content had a significantly lower (n=30, $p \leq 0.001$) elastic modulus **(Figure 4.9)**, significantly lower (n=30, $p=0.005$) ultimate stress **(Figure 4.10)** and a significantly higher (n=30, $p=0.003$) tendon CSA **(Figure 4.11)**. Tendons containing a higher GAG content had a

significantly higher (n=30, p=0.028) tendon CSA **(Figure 4.12)**. Results of collagen fibril diameters showed that horses with a higher MAD had a significantly lower (n=13, p=0.005) DNA content **(Figure 4.13)**.

There were no significant correlations found between CFI and material properties or matrix composition in this study. There were no significant correlations found for collagen content with material properties, collagen fibril diameters, or matrix composition.

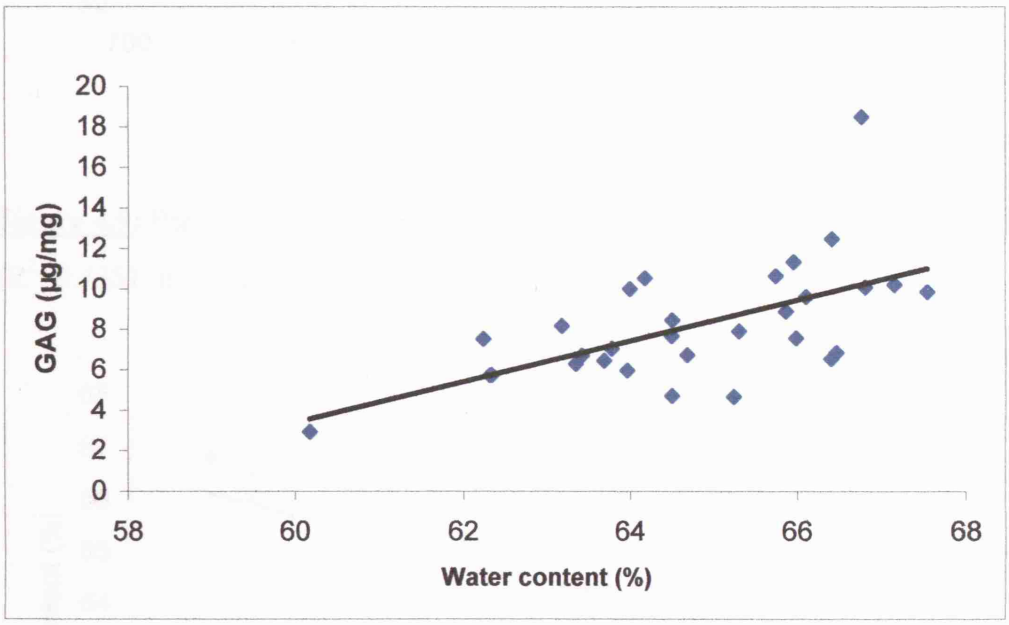


Figure 4.8: Total sulphated glycosominoglycan (GAG) content in the SDFT against percentage water content (R^2=0.3553, n=30, p≤0.001).

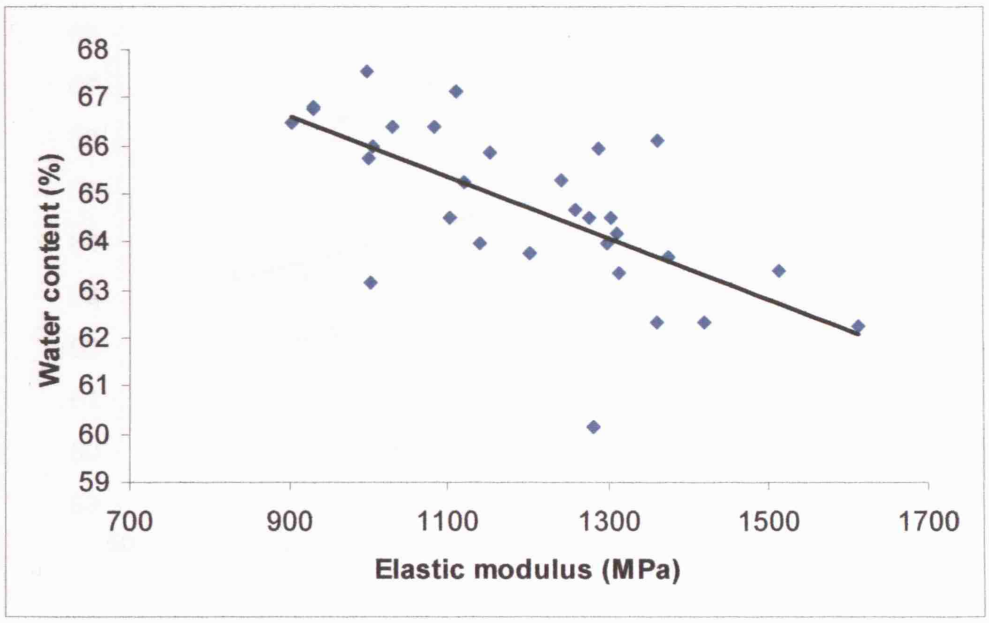


Figure 4.9: Percentage water content in the SDFT against elastic modulus (R^2=0.4351, n=30, p≤0.001).

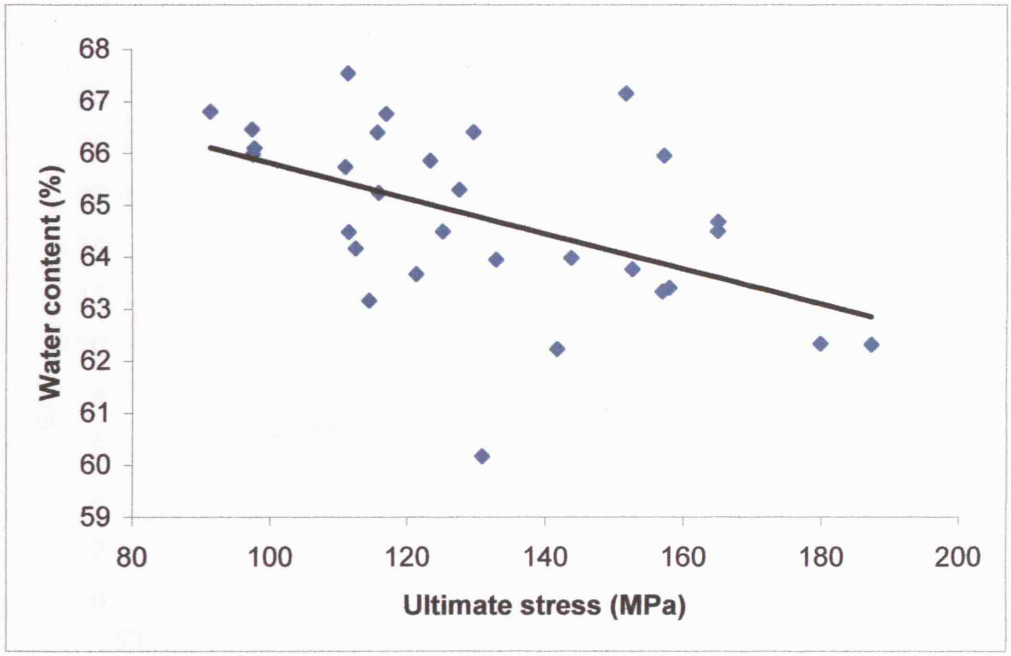


Figure 4.10: Percentage water content in the SDFT against ultimate stress (R^2=0.2484, n=30, p=0.005).

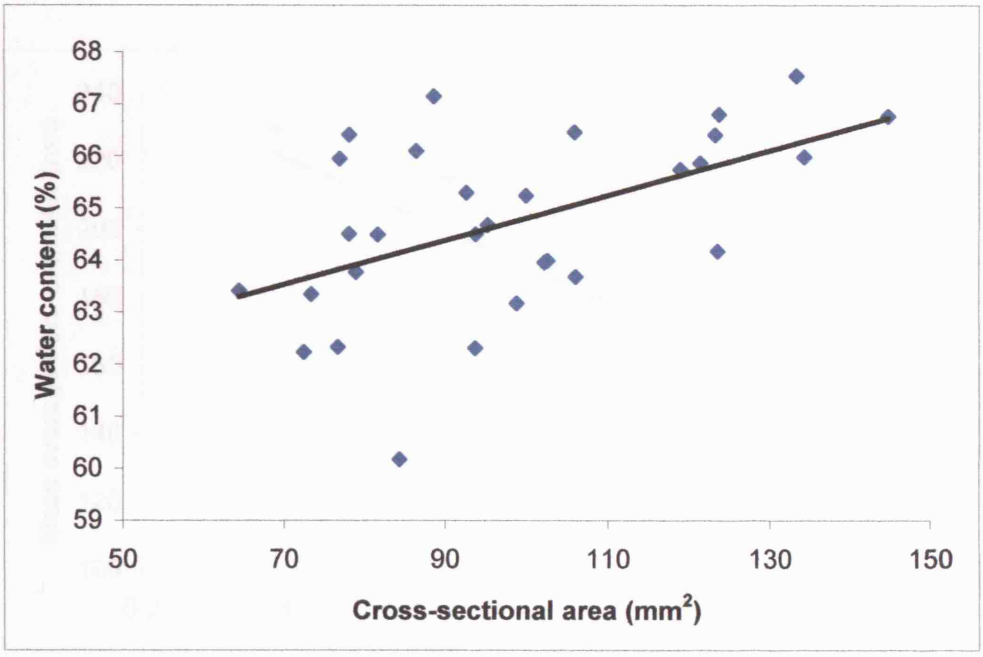


Figure 4.11: Percentage water content in the SDFT against tendon CSA (R^2=0.2761, n=30, p=0.003).

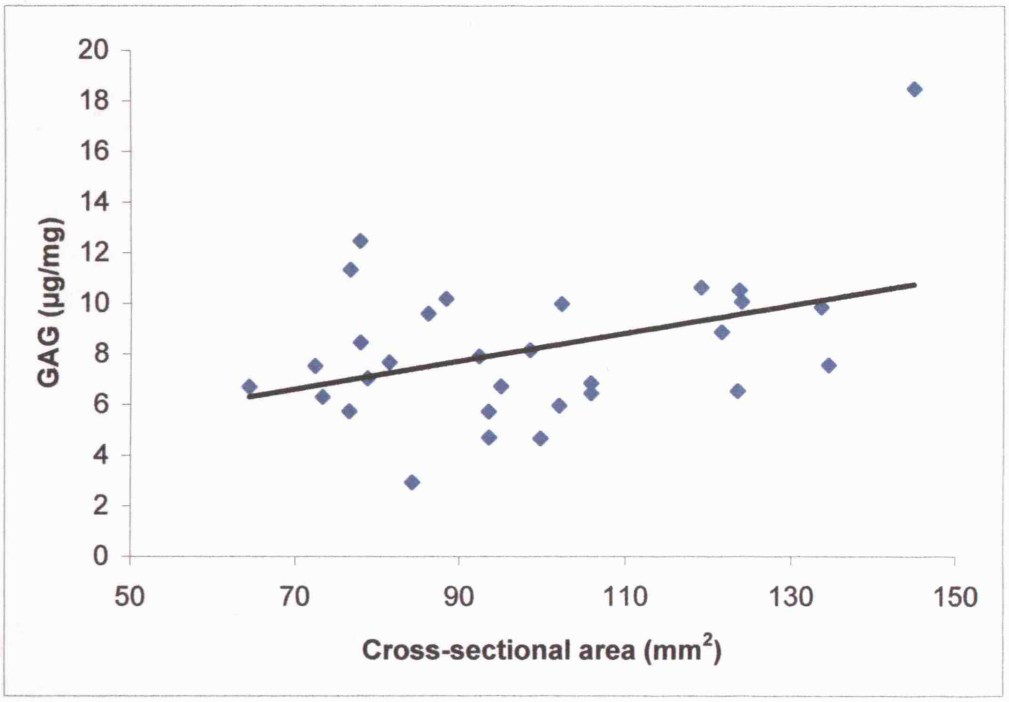


Figure 4.12: Total sulphated glycosaminoglycan (GAG) content in the SDFT against tendon cross-sectional area (CSA). (R^2=0.1615, n=30, p=0.028).

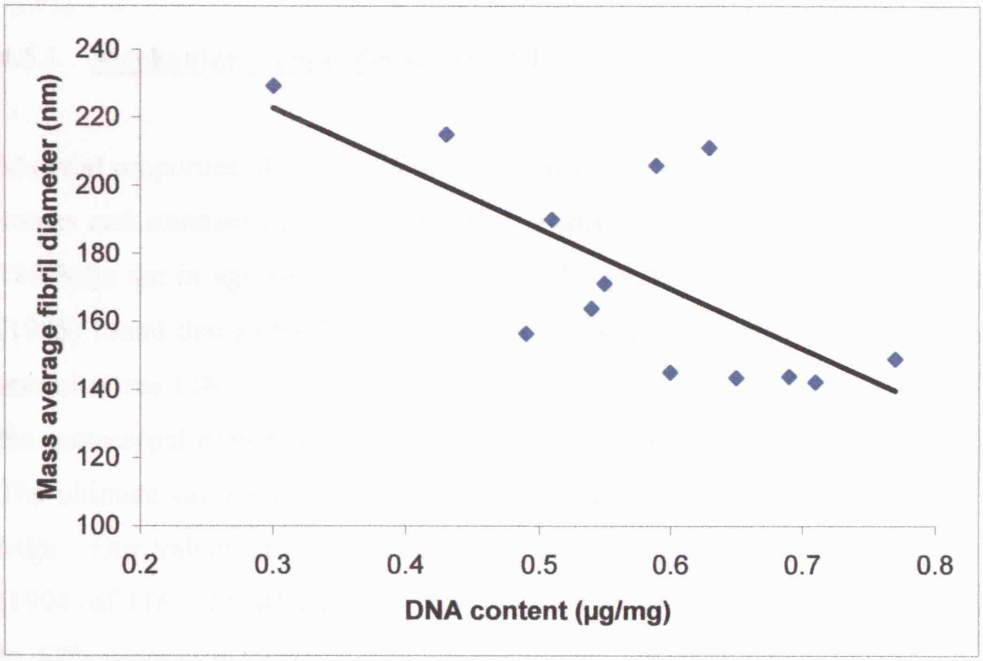


Figure 4.13: Mass average collagen fibril diameter in the SDFT against deoxyribonucleic acid (DNA) content (R^2=0.4872, n=13, p=0.005).

4.6 DISCUSSION

4.6.1 Mechanical Properties of the SDFT

Material properties of the SDFT showed a wide variation between horses. The mean values and standard deviations obtained in this study for elastic modulus of 1197 ± 180 MPa are in agreement with those of other authors. Riemersma & Schamhardt (1985) found that in the hindlimb of the SDFT in the metatarsal region, the elastic modulus was 1282 ± 127 MPa. Crevier *et al.* (1996) measured the elastic modulus in the metacarpal region of the forelimb SDFT and found this to be 1189 ± 63 MPa. The ultimate stress values of the SDFT obtained in the present study were 132 ± 25 MPa. Our values are slightly higher than those reported by Jansen & Savelberg (1994) of 116 ± 21 MPa and Crevier *et al.* (1996) of 109 ± 8 MPa which may be due to differences in mechanical testing procedures.

There is a postitive relationship between the material properties of ultimate stress and elastic modulus in tendon. Tendons that are made of stiffer material are also made of stronger material which may have an effect on the elastic energy storage role and strain in relation to peak forces. The significant positive correlation between elastic modulus and ultimate stress found in this study is in agreement with that of Birch *et al.* (2000) who evaluated the right to left symmetry of the SDFT in terms of mechanical stiffness and strength. As the elastic modulus of tendon increases, the ultimate stress also increases, so therefore tendons that have a high elastic modulus will have a high ultimate stress. Understanding the underlying mechanisms that result in stronger and stiffer tendons may be important in relation to achieving optimal tendon function. It is therefore also important to determine those characteristics of the matrix which can predict strong and weak tendons.

The data obtained in the present study for ultimate tensile forces **(Figure 4.1)** suggest that some horses may have weaker tendons, which predispose to tendon overload and subsequent injury. However, this may depend on the applied forces as a function of body weight and speed with some effect of the muscle in terms of tuning to maximise the energy storage. In the present study, the ultimate strength values demonstrate a normal distribution but a wide range between horses, which is in

agreement with the work of Wilson (1991). In the future, the ability to identify such horses that have weaker tendons may enable individuals to be protected from excessive levels of exercise and therefore avoid a painful and disabling tendon rupture. The selection of horses with tendons having appropriate mechanical properties could be important in maximising performance and minimising injury. It is not yet possible to predict which animals have tendons with exceptionally high or low strengths or stiffnesses. However, if further research allows such predictions, it may be possible to identify horses at risk of tendon injury prior to training.

The data obtained in the present study for % ultimate strain, indicate that some tendons broke at twice the ultimate strain of others illustrating that there is a wide variation in the mechanical properties of different horses and mechanical properties are specific to an individual. Previous research has shown that the entire SDFT fails at a strain of 12-20 % (Riemersma & Schamhardt, 1985, Wilson, 1991). Our range begins lower possibly due to the fact that 7 tendons pulled out of the top clamp during mechanical testing; otherwise our results are in agreement with the work of others.

4.6.2 Structural Properties of the SDFT and the Relationship to Material Properties

The SDFT showed considerable horse to horse variation in CSA (98 ± 21 mm^2, n=30). Values obtained in this study are similar to those measured in other studies. Birch *et al.* (1999a) obtained a value of 101 ± 16 mm^2 for the CSA of the mid-metacarpal region of the SDFT on post mortem specimens. Crevier *et al.* (1996) used ultrasound on post mortem specimens and a value of 113 ± 13 mm^2 was obtained in the mid-metacarpal region of the SDFT. Smith *et al.* (1994) using ultrasound in the live animal, recorded values of 77 – 177 mm^2 for the mid-metacarpal region of the SDFT in thoroughbred horses. In the present study our range in CSA for the SDFT of 64 – 145 mm^2 is similar to those values obtained in the live animal.

The results show that larger tendons had a lower elastic modulus in the SDFT. This may be because some horses have weaker tendons than others which is supported by

our data. Tendons with a larger CSA are stiffer and stronger. SDFTs with a lower elastic modulus are less able to function effectively in their role as energy storing tendons. Those tendons often either injured or predisposed to injury appear to be less elastic and may therefore be more prone to injury.

4.6.3 Collagen Fibril Diameters and the Relationship to Material Properties

The values obtained in this study for MAD of 169 ± 25 nm in the SDFT are slightly higher than those found by Patterson-kane *et al.* (1997a) of 131.7 ± 4.9 nm. This may be due to differences in experimental technique in relation to tissue processing and measurement of collagen fibrils. The values obtained in the present study for CFI of 61.2 ± 4 % are lower than those found by Patterson-kane *et al.* (1997a) of 71.7 % in the SDFT of nonexercised thoroughbreds, but similar to 66.2 % found in exercised Thoroughbreds. Our study shows that CFI values are also lower than those found by Cherdchutham *et al.* (2001) who measured collagen fibril diameters in foals. Again, this is may be due to differences in experimental technique in relation to tissue processing and measurement of collagen fibrils. In our study there was a significant positive correlation between CFI and MAD but the CFI did not correlate with other mechanical properties such as tendon CSA, elastic modulus and ultimate stress. Parry & Craig (1988) have shown that the CFI correlates with *in vitro* ultimate strength of tendon. Possibly the reason why we did not find this trend is due to the fact that we only measured the CFI in the SDFTs of 10 horses. Perhaps if we had collected more data on CFI we would have found a similar trend as a reverse power calculation would be required to determine how many horses were needed for this.

The collagen fibril diameters were related to the material properties of the tendon. There was a significant positive correlation between MAD and elastic modulus of the tendon. SDFTs which have a lower elastic modulus and are therefore more easily stretched, contain smaller collagen fibrils and therefore have a lower MAD. Other studies have also found that the MAD has been positively correlated with the *in vitro* ultimate strength of tendon (Oakes 1989; Parry & Craig 1988). Parry *et al.* (1978b) hypothesised that large diameter fibrils have greater tensile strength due to the higher density of intrafibrillar covalent cross-linkages between collagen molecules which

prevents slippage between the latter when the fibril is loaded. This means that at the same level of increasing strain, a small diameter fibril fails before a large diameter fibril. A reduction in the number of large fibrils and an increase in the number of small fibrils would be expected to result in a decrease in elastic modulus. If a tendon continues to increase in CSA then it would be expected that the MAD will decrease resulting in a larger number of small diameter fibrils. This increase in the number of small diameter fibrils may reduce tendon tensile strength in the core region, making it more prone to injury and development of degenerative core lesions. A high MAD indicates stronger structural properties in the SDFT, which would be expected to have a higher resistance to injury. Determining the mechanisms that control fibrillogenesis is now a key area to focus on and may be important in further understanding the relationships between tendon structure and mechanical properties.

4.6.4 Matrix Composition and the Relationship to Material Properties

Our results showed that the elastic modulus had a significant negative correlation with the water content of the tissue, therefore tendons with higher water content are less stiff. The water content showed a significant positive correlation with the total sulphated glycosaminoglycan content and tendon CSA in the SDFT. Both water content and glycosaminoglycan content can be assessed using magnetic resonance imaging (MRI) (Kasashima *et al.* 2002a), therefore the prediction of mechanical properties *in vivo* could be possible. In the future, this study could provide a basis for the selection of potential biochemical markers present in blood, urine or tendon synovial sheath fluid samples which may be useful in monitoring tendon functional integrity. The mechanical properties of the tendon did not relate simply to total collagen content. However the organisation of the collagen component appears important in determining mechanical properties in terms of the collagen fibril morphology.

In the present study there were some outliers. The first horse was a 16 year old mare, 16.3 hands high (hh), Irish Thoroughbred - Ex show jumper (B Grade) who had show jumped until 3 months before the horse had been destroyed for reasons relating to old age. This horse had the lowest water content of 60.2 % in the SDFT and the highest MAD value of 229 nm. This horse was the only show jumper in the study,

therefore, the fact that it was a different type of horse and had undergone a different type of exercise to the other horses may have contributed to it being an outlier. Also, this horse was a heavier type of horse and show jumpers rarely injure the SDFT, as tendon injury is more common in the SL. The second outlier horse was a 15 year old Thoroughbred gelding, which had an exercise history of ex point to point racing and had won lots of races. This horse had been destroyed for lameness. This horse had the highest tendon CSA in the SDFT of 145 mm^2 and the highest total sulphated GAG content of 18.5 μg/mg. The left SDFT was slightly larger than the right SDFT in terms of tendon CSA, which relates to the fact that this horse had the highest GAG content. This horse may have had a tendon injury to the left SDFT since it was also lame.

4.7 CONCLUSIONS

- SDFTs which have a lower elastic modulus and are therefore more easily stretched, have a similar collagen content to SDFTs made of a stiffer material but have smaller diameter collagen fibrils.

- SDFTs which have a lower elastic modulus and are therefore more easily stretched, have a higher water content and higher glycosaminoglycan (GAG) content than SDFTs made of a stiffer material but have smaller diameter collagen fibrils.

- Larger SDFTs, in terms of cross-sectional area, have a lower elastic modulus (i.e. have a less stiff matrix) and appear to be less able to function effectively in their role as energy storing tendons due to the fact that these horses may have weaker tendons and are more prone to injury.

- An understanding of the material properties of normal tendon is an important prerequisite for the study of tendon degeneration. The correlations with morphology, matrix composition, and material properties found in this study provide an appreciation of the complexity and functional abilities of tendon.

CHAPTER 5

IS THE SPECIFIC FUNCTION OF A TENDON REFLECTED IN ITS MATERIAL PROPERTIES?

5.1 INTRODUCTION

5.1.1 Function of Specific Tendons

Tendons have specific functions, which are important in the role they play in locomotion. In the horse, the SDFT and SL play a significant role in storing and releasing elastic energy. For this energy to be of use to the horse it must be released at an appropriate rate which will depend on the stiffness of the structure. The SDFT needs to be highly elastic, i.e. have a low stiffness, in order to stretch and return a maximum amount of stored energy. The SDFT is loaded early in the stance phase and experiences high stresses and strains during high-speed locomotion. The DDFT, in contrast, is loaded later in the stance phase, is subjected to lower stresses and strains (Platt *et al.,* 1994), and is therefore much less important for energy storage. The SL plays a role in energy storage for locomotion along with the flexor tendons (Goodship & Birch, 2001).

Other tendons, such as the CDET function as non-energy storing tendons. The CDET positions the foot prior to placement, and functions merely to transfer force created by the muscle to the bone and make joint movement possible. These tendons need to be stiff enough not to allow elongation to take place.

The horse is an ideal model in which to investigate the different functions of tendons in relation to differences in mechanical properties and morphology, which can also be applied to human tendons. The horse undertakes athletic activity and the tendons are part of the locomotor system, and as such a component of energetic efficiency mechanisms. The equine SDFT and human Achilles tendon are both subject to the effects of competitive athletic activities and play a similar functional role as energy storing tendons, and both sustain high rates of injury. This was reported by Goodship & Birch (2001) who observed very similar injuries in the human Achilles tendon when compared to lesions in the SDFT of the horse. In comparison, the equine CDET and human tibialis anterior tendons have similar functional roles as positional tendons and rarely sustain injury.

5.1.2 Mechanical properties of tendons with specific functions

Different tendons have specific requirements in terms of their mechanical stiffness to ensure normal physiological function. Flexor tendons have mechanical properties that generally make them well suited to act as biological springs. Some flexor tendons are relatively stiff (i.e. have a high elastic modulus), can sustain high tensile stress, can stretch elastically to low strains and are very resilient (Bennett *et al.*, 1986; Ker 1981; Woo *et al.*, 1982). These studies were carried out on mammals such as the sheep and swine. It has also been reported that the % strain can be much higher for some tendons, in particular, those of the horse. The SDFT, DDFT, and SL, have been shown to have *in vitro* ultimate % strain values of 10-12 % (Jansen & Salvelberg, 1994; Riemersma & Schamhardt, 1985).

Different mechanical properties are evident in tendons that have different functional roles. A comparison of mechanical properties of digital tendons in mature swine showed that flexor tendons were significantly stronger and stiffer than extensor tendons and displayed lower mechanical hysteresis and are therefore better suited to act as effective biological springs (Shadwick, 1990). Birch *et al.* (2001) and Batson *et al.* (2003) demonstrated that tendons which function as an elastic energy store, such as the human Achilles tendon and equine SDFT, have a lower elastic modulus than non-energy storing tendons. In contrast, bone is stiffer as a material than tendon and has a higher elastic modulus although it does not function as an elastic energy store.

Riemersma & Schamhardt (1985) carried out a study on *in vitro* mechanical properties of the SDFT, DDFT, and SL hindlimb tendons of the horse. It was found that large differences in elastic modulus exist between different tendons and that these differences correspond with differences in CSA and collagen content. There are limitations of this study however, because although this information is interesting, the study was conducted on hindlimb tendons, which do not have as high an incidence of injury as the distal forelimb tendons. Also, only flexor tendons and energy storing tendons were studied so it would be useful to compare these to non-energy storing tendons. To date, no study has compared the material properties directly to the collagen fibril diameters and matrix composition of the SDFT, DDFT,

SL, and CDET equine distal forelimb tendons, so this will be the main focus of the present study.

5.1.3 Morphological variation in tendons with specific functions

Differences exist in collagen fibril diameter distributions between specific tendons in the horse (Patterson-Kane *et al.,* 1997a). For example, the SDFT and SL show a relatively high proportion of small diameter collagen fibrils in comparison to the DDFT. It is thought that smaller diameter collagen fibrils in the SDFT with respect to the DDFT may provide the SDFT with greater elasticity (Birch *et al.,* 1999b).

5.1.4 Composition of tendons with specific functions

Interestingly, the composition of the extracellular matrix in terms of the macromolecules and the way in which these are organised also play a role in determining tendon mechanical properties (Birch *et al.,* 1999a). Differences in macromolecular composition exist between equine flexor tendons. In a study by Birch *et al.* (1999b) it was shown that differences in composition exist between the SDFT and DDFT, which may reflect their different mechanical roles *in vivo.* Although the percentage collagen content was the same for both tendons, there were substantial differences in the collagen type and organisation. The SDFT had a higher type III collagen content, lower total chondroitin sulphate equivalent glycosaminoglycan content, smaller diameter collagen fibrils and a higher cellularity than the DDFT.

5.2 Hypothesis

Tendons with a higher elastic modulus (stiffer) have larger collagen fibril diameters and lower water and sulphated GAG contents.

5.3 Objectives

1. To determine structural and material properties, collagen fibril morphology, and matrix composition of energy storing structures (SDFT, SL) and non-energy storing structures (DDFT, CDET) from the equine distal forelimb.

2. To investigate whether relationships exist between matrix composition, collagen fibril morphology and material properties in these tendons that have different functions.

5.4 Experimental Design

In this part of the study structural and material properties of the SDFT, DDFT, SL and CDET will be determined on left forelimb tendons. In particular the tendon cross-sectional area, ultimate load, % ultimate strain, elastic modulus, and ultimate stress will be measured. The organisation of collagen in the matrix is important (refer to Chapter 1) and this will be assessed by measuring collagen fibril diameters in the right forelimb SDFT, DDFT, SL and CDET. The relationship between material properties and fibril diameters can then be investigated. This will allow an investigation into whether tendons are designed for a specific function, which is reflected in their material properties and morphology, to be carried out. In terms of matrix composition, the water content, GAG content, and collagen content will be measured in the right forelimb SDFT, DDFT, SL and CDET so that correlations with collagen fibril morphology and material properties can be determined. Qualitative assessment will also be made of histological transverse sections to investigate fascicle morphology between the energy storing SDFT and the non-energy storing CDET.

5.5 RESULTS

5.5.1 Cross-sectional area

Tendon CSA was significantly different ($p \leq 0.001$) between the SDFT, DDFT, SL and CDET **(Figure 5.1)**. Tendon CSA measurements showed that the mean value of the SL (192 ± 48 mm^2) was the largest, followed by the DDFT (167 ± 41 mm^2), SDFT (100 ± 16 mm^2), and the CDET (30 ± 7 mm^2) was the smallest **(Table 5.1)**. The CSA of the SDFT was significantly higher than the CDET (n=6, $p \leq 0.001$), but significantly lower than the DDFT (n=6, $p \leq 0.001$) and the SL (n=6, $p \leq 0.001$) **(Table 5.1, Figure 5.1)**.

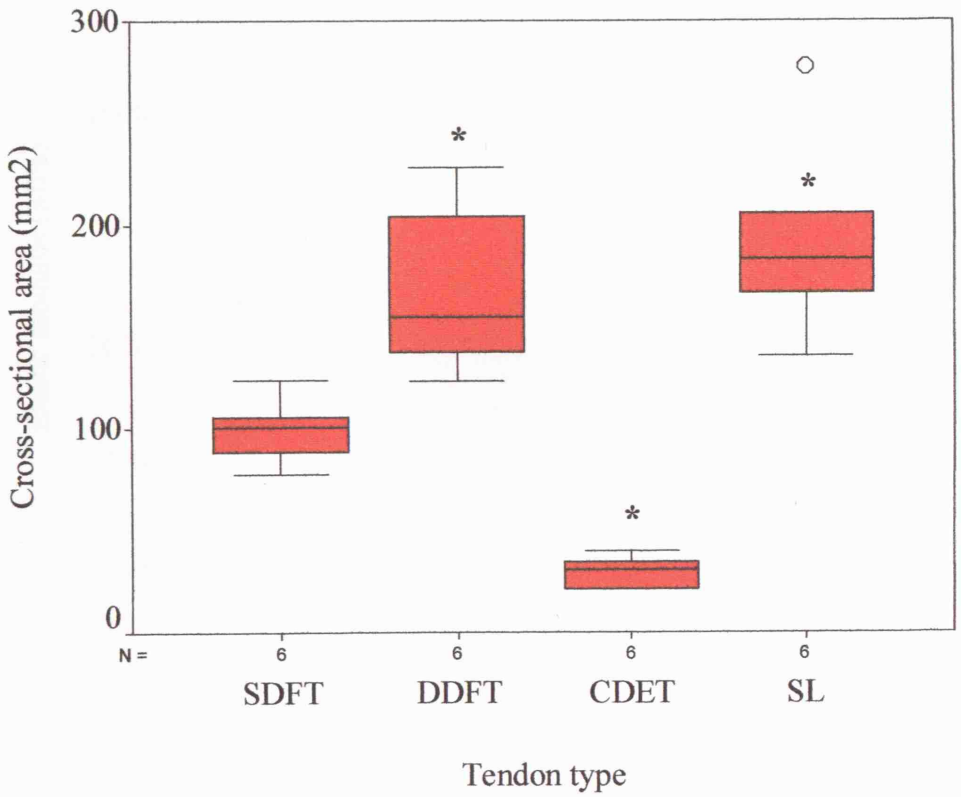


Figure 5.1: Boxplot showing tendon CSA values for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. o represents an outlier value, * Denotes a significant difference relative to the SDFT).

5.5.2 Material and Structural Properties

The material properties were significantly different between the different structures **(Table 5.1)**. There was a significant difference in elastic modulus between the SDFT, DDFT, SL and CDET ($p \leq 0.001$). Elastic modulus results showed that the mean value of the CDET (1510 ± 291 MPa) was the highest followed by the SDFT (1165 ± 178 MPa), DDFT (823 ± 195 MPa), and the SL (643 ± 130 MPa) was the lowest **(Table 5.1)**. The elastic modulus of the SDFT was significantly higher than that of the DDFT (n=6, p=0.005), and the SL (n=6, $p \leq 0.001$), but significantly lower than that of the CDET (n=6, p=0.005). The DDFT and SL were not significantly different in terms of elastic modulus **(Figure 5.2)**.

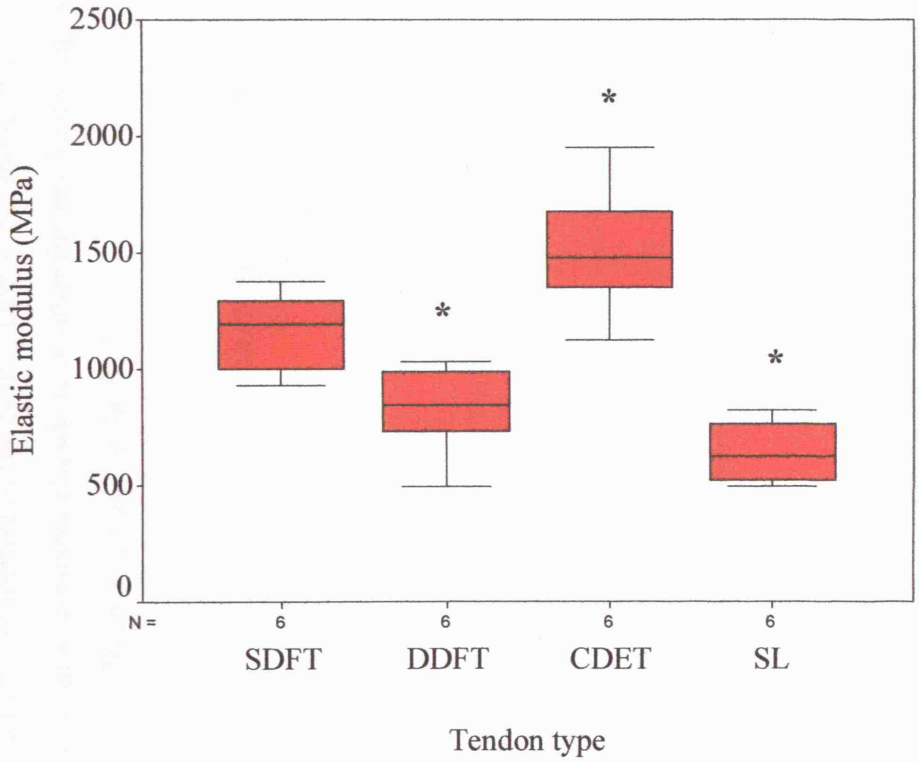


Figure 5.2: Boxplot showing elastic modulus values for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* Denotes a significant difference relative to the SDFT.

Table 5.1: Structural and material properties of the SDFT, DDFT, SL and CDET.

This table summarises information available elsewhere in other figures from Chapter 5.

(n=6, * denotes a significant difference relative to the SDFT).

Tendon	Tendon CSA (mm^2)	Ultimate Load (kN)	Ultimate Strain (%)	Elastic Modulus (MPa)	Ultimate Stress (MPa)
SDFT	100 ± 16	12.8 ± 1.3	14.5 ± 2.6	1165 ± 178	131 ± 27
DDFT	167 ± 41 *	16.5 ± 3.1	19.8 ± 5.1	823 ± 195 *	100 ± 19
SL	192 ± 48 *	16.8 ± 2.5 *	20.2 ± 5.2	643 ± 130 *	91 ± 19 *
CDET	30 ± 7 *	5.5 ± 1.5 *	18.5 ± 5.8	1510 ± 291 *	189 ± 37 *

The ultimate stress was significantly different between the SDFT, DDFT, SL and CDET ($p \leq 0.001$). Ultimate stress results showed that the mean value of the CDET (189 ± 37 MPa) was the highest followed by the SDFT (131 ± 27 MPa), DDFT (100 ± 19 MPa), and the SL (91 ± 19 MPa) was the lowest **(Table 5.1)**. The ultimate stress of the SDFT was significantly lower than the CDET (n=6, p=0.004) but significantly higher than the SL (n=6, p=0.04). The ultimate stress of the DDFT was not significantly different from either the SDFT or the SL **(Figure 5.3)**.

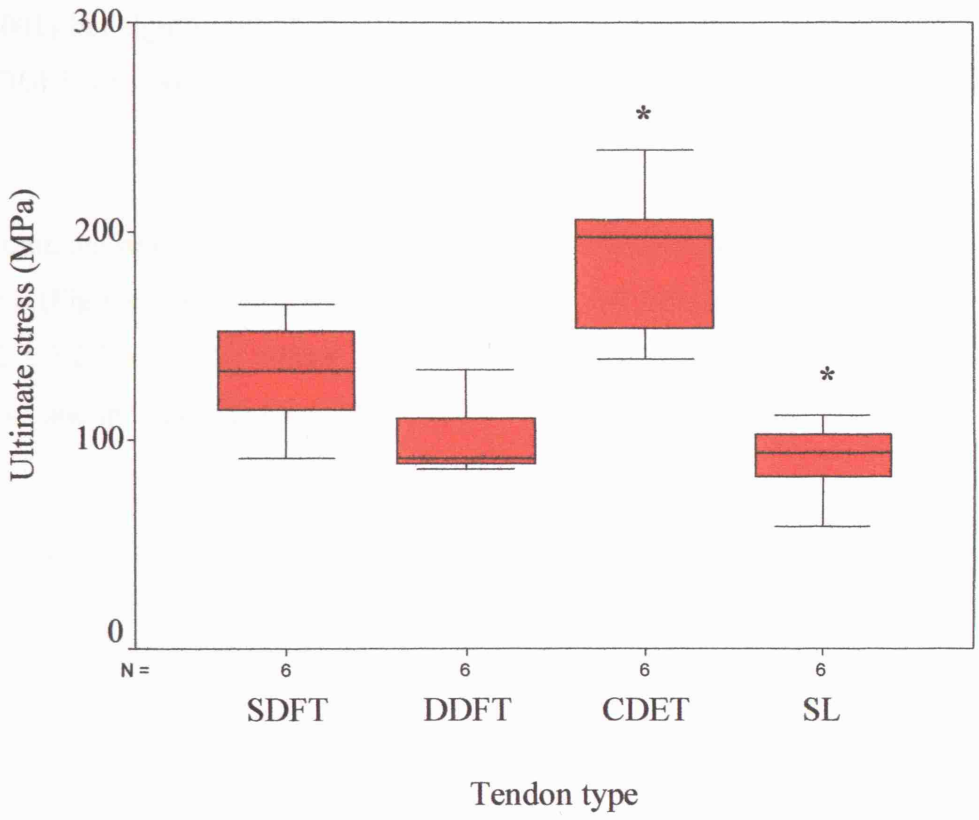


Figure 5.3: Boxplot showing ultimate stress values for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* Denotes a significant difference relative to the SDFT.

Load deformation plots of the SDFT, DDFT, CDET, and SL from one of the horses are shown in **Figure 5.4**. It can be seen that the slopes of the curves differ between tendons.

In terms of structural properties, the ultimate load was significantly different between the SDFT, DDFT, SL and CDET ($p \leq 0.001$). Ultimate load results showed that the mean value of the SL (16.8 ± 2.5 kN) was the highest, followed by the DDFT (16.5 ± 3.1 kN), SDFT (12.8 ± 1.3 kN), and the CDET (5.5 ± 1.5 kN) was the lowest **(Table 5.1)**. The ultimate load of the SDFT was significantly higher than the CDET ($n=6$, $p \leq 0.001$) but significantly lower than the SL ($n=6$, $p=0.048$). The ultimate load of the DDFT was not significantly different from either the SDFT or the SL **(Figure 5.5)**.

The ultimate strain was not significantly different between the SDFT, DDFT, SL and CDET **(Figure 5.6)**. Ultimate strain results showed that the mean value of the SL (20.2 ± 5.2 %) was the highest, followed by the DDFT (19.8 ± 5.1), CDET (18.5 ± 5.8 %), and the SDFT (14.5 ± 2.6 %) was the lowest **(Table 5.1)**.

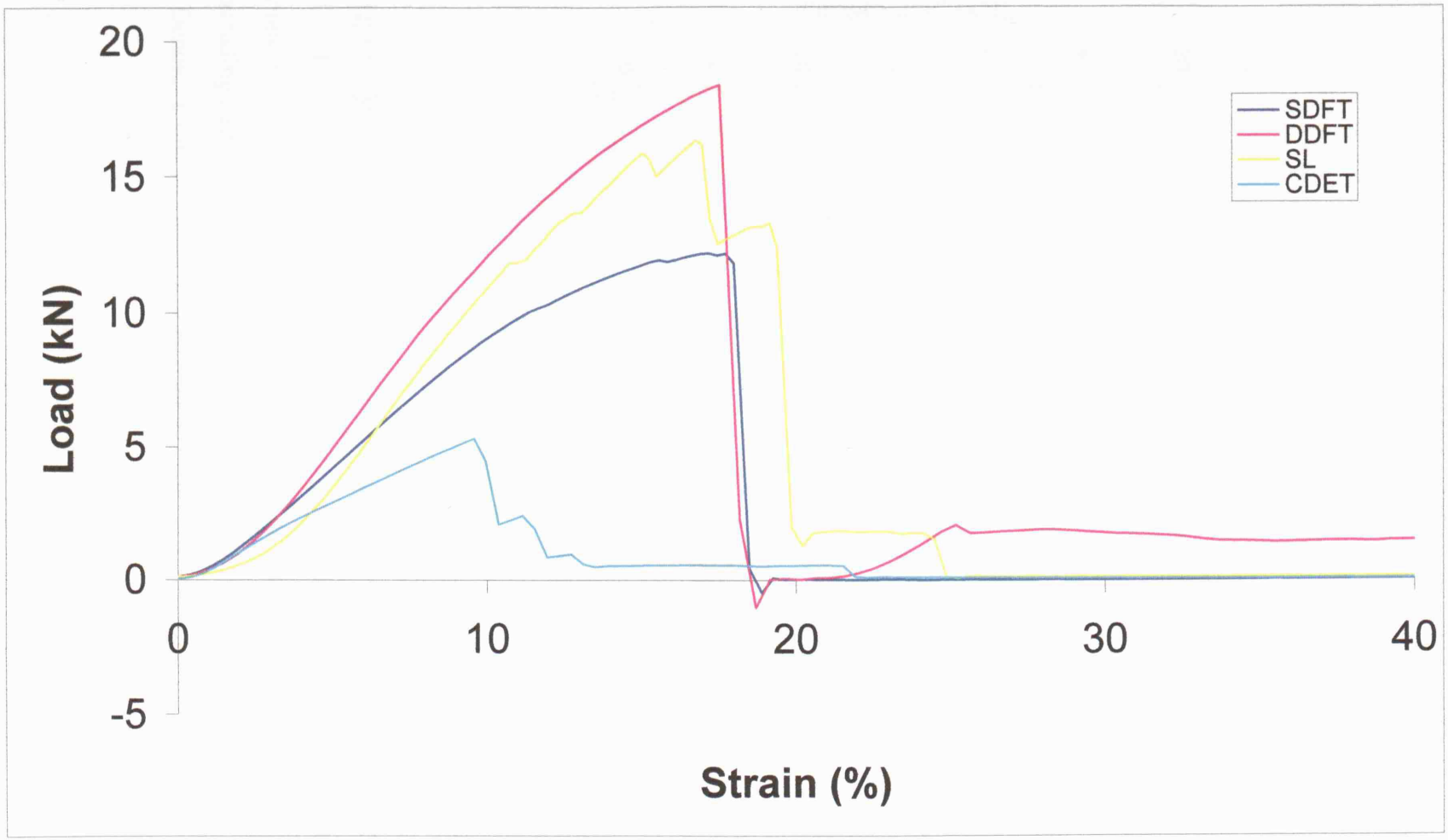


Figure 5.4: Load deformation plot for SDFT, DDFT, SL, and CDET from one horse showing ultimate load variation between tendons.

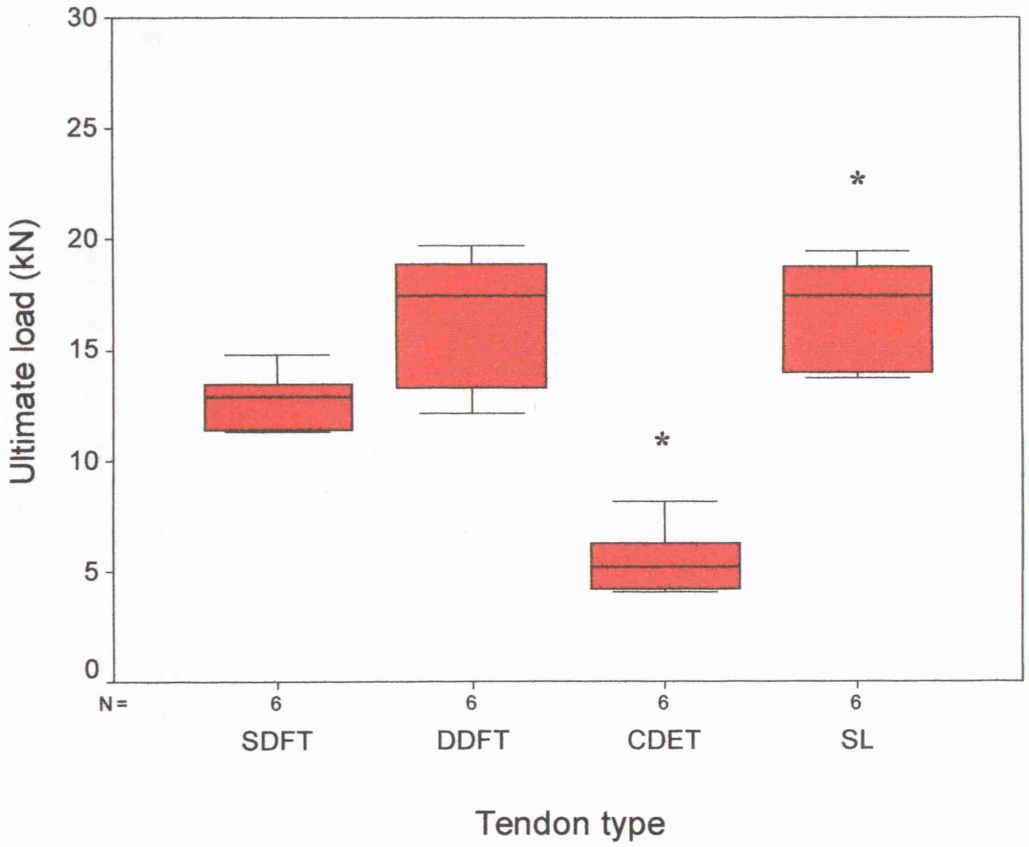


Figure 5.5: Boxplot showing ultimate load values for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* Denotes a significant difference relative to the SDFT.

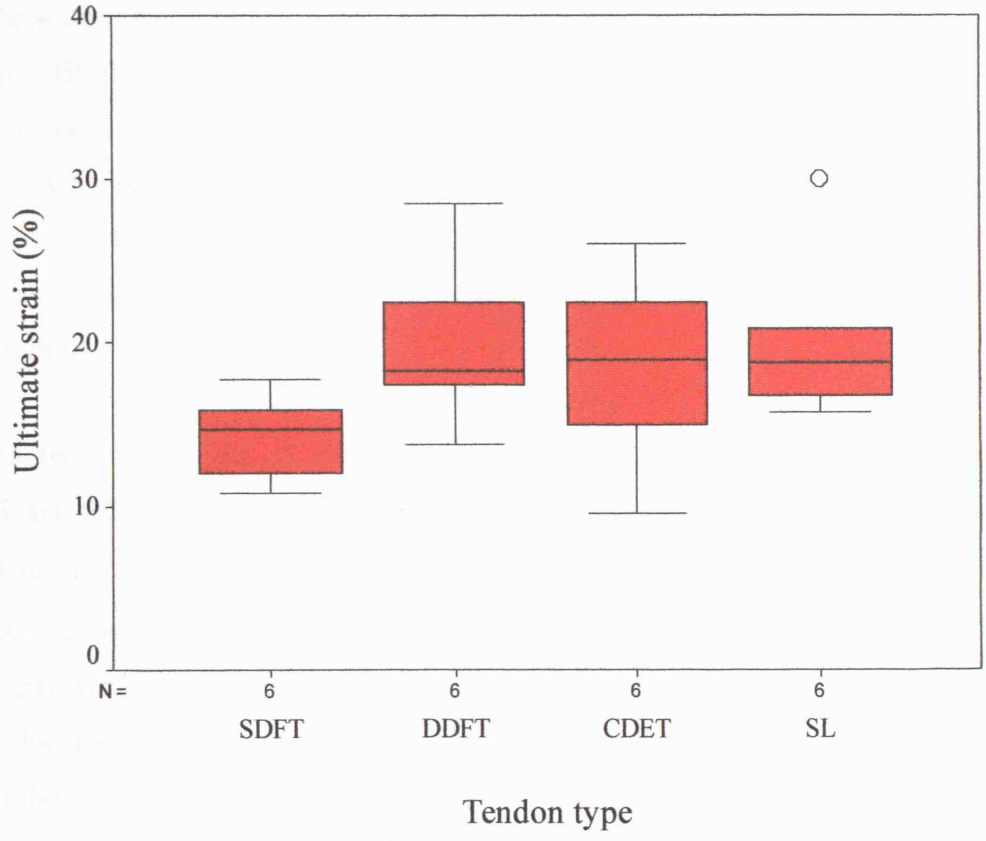


Figure 5.6: Boxplot showing ultimate strain values for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. o represents an outlier value).

5.5.3 Collagen Fibril Diameters

Collagen fibril diameters ranged from 11 - 459 nm and the SDFT, DDFT, SL and CDET all showed a bimodal distribution **(Figure 5.7)**. Collagen fibrils, in terms of numbers, with a diameter less than 160 nm represented 83 % of the total in the SDFT, 66% of the total in the DDFT, 58% of the total in the CDET, and 96 % of the total in the SL. Qualitative assessment of the electron micrographs **(Figure 5.8)** showed that the large collagen fibrils occupied most of the area in the CDET, and that there were small fibrils present between the large ones. In the DDFT the large collagen fibrils also occupied most of the area, with smaller fibrils present in between the large ones. The SDFT showed considerably more small fibrils to be present in between the large fibrils and the area of the SL was occupied mainly by small fibrils with considerably less large ones. A quantitative assessment of the collagen fibril diameters was then carried out, which supported these qualitative observations.

Quantitative assessment of the collagen fibril diameters showed there was a significant difference in MAD between the SDFT, DDFT, SL and CDET ($p \leq 0.001$). MAD results showed that the mean value of the CDET (229 ± 36 nm) was the largest, followed by the DDFT (215 ± 32 nm), SDFT (169 ± 19 nm), and the SL (122 ± 14 nm) was the smallest **(Table 5.2)**. The MAD of the SDFT was significantly lower than the CDET ($n=6$, $p=0.002$) and the DDFT ($n=6$, $p=0.018$), but significantly higher than the SL ($n=6$, $p=0.017$). The DDFT and the CDET were not significantly different in terms of MAD **(Figure 5.9)**.

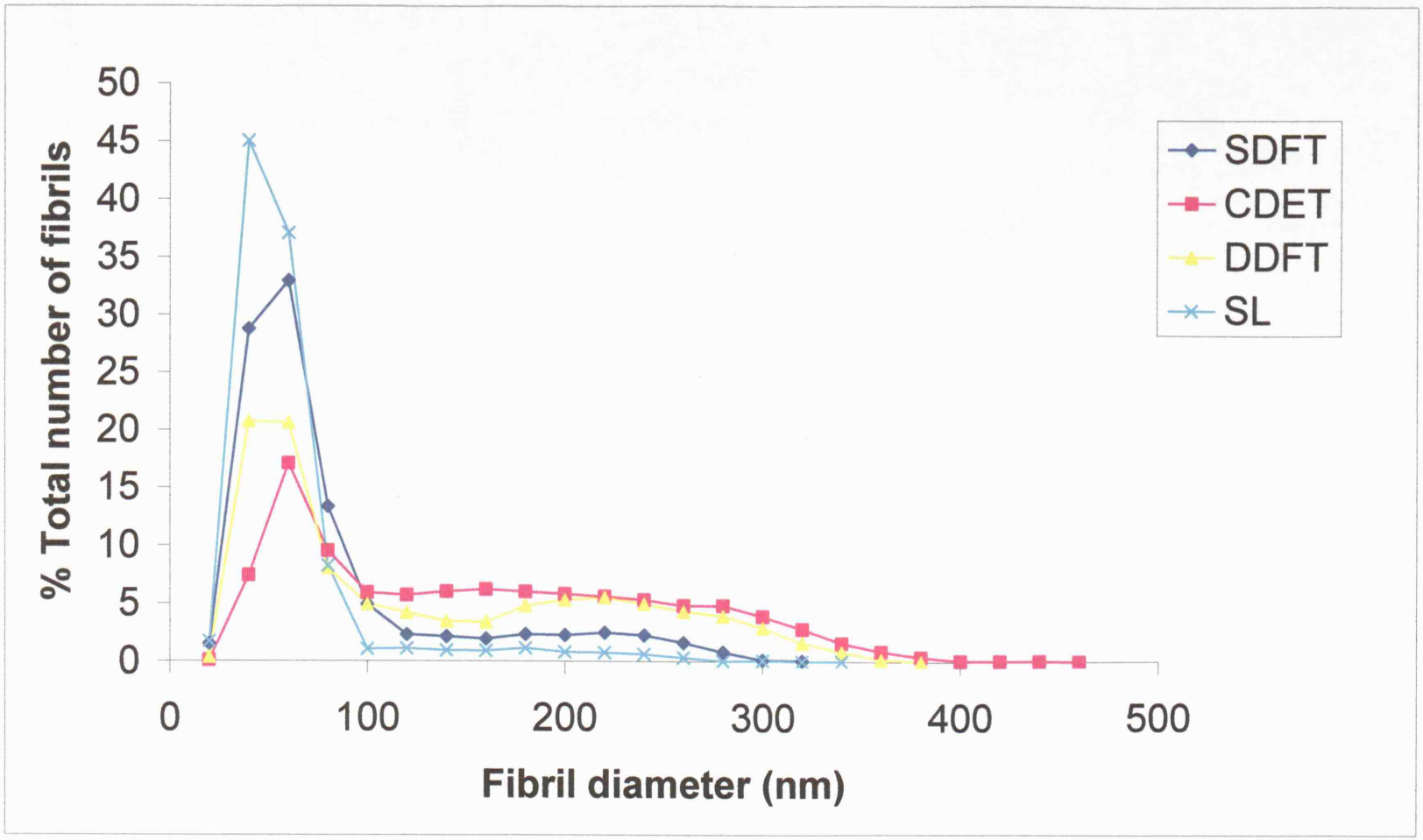
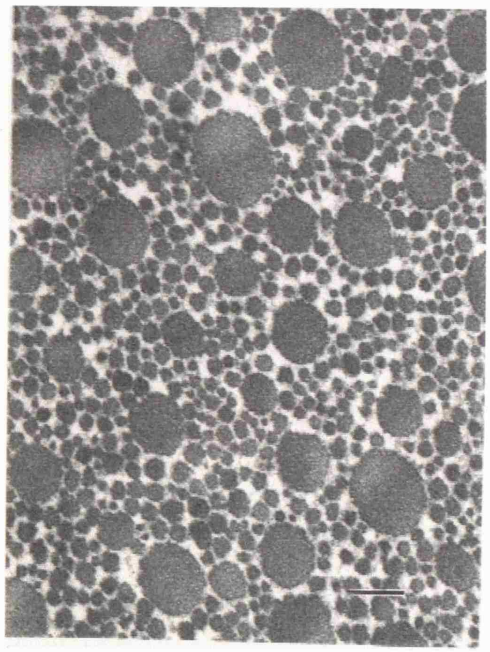
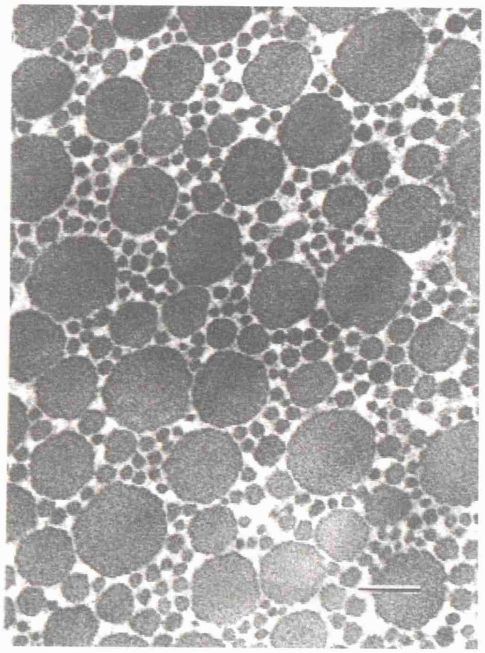


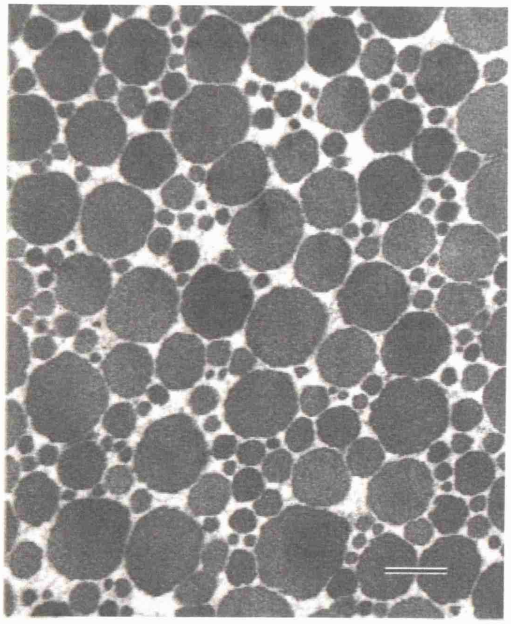
Figure 5.7: Collagen fibril diameter distribution of the SDFT, DDFT, SL, and CDET.



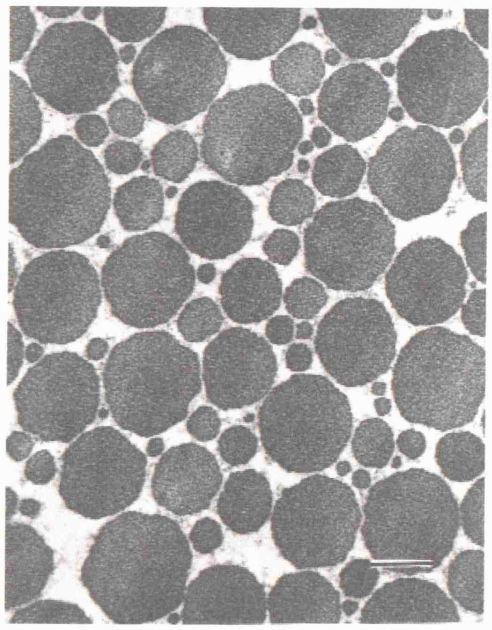
SL



SDFT



DDFT



CDET

Figure 5.8: Electron micrographs of SDFT, SL, DDFT and CDET collagen fibrils. (Bar represents 200 nm).

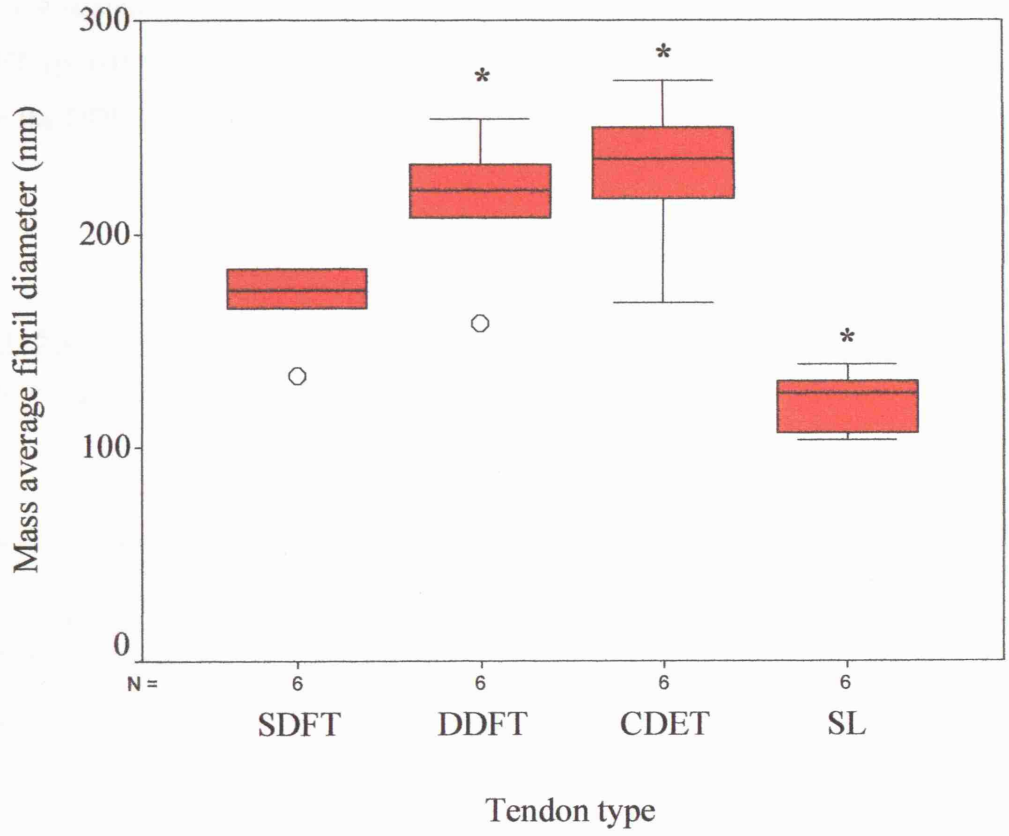


Figure 5.9: Boxplot showing mass average fibril diameters for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. o Represents an outlier value, * Denotes a significant difference relative to the SDFT).

5.5.4 Matrix Composition

The water content was significantly different **(Figure 5.10)** between the different structures ($p \leq 0.001$). Water content results showed that the mean value of the SL (68 ± 0.7 %) was the highest, followed by the SDFT (65 ± 1.7 %), DDFT (62 ± 1.4 %), and the CDET (57 ± 0.6 %) was the lowest **(Table 5.2)**. The water content in the SDFT was significantly lower than the SL ($p=0.035$) but significantly higher than the CDET ($p \leq 0.001$). The water content in the SDFT was not significantly different from the DDFT **(Figure 5.10, Table 5.2)**.

Table 5.2: Matrix and morphological properties of the SDFT, DDFT, SL and CDET. (n=6, * denotes a significant difference relative to the SDFT).

Tendon	Water Content (%)	GAG Content (μg/mg)	Collagen Content (%)	Mass Average Fibril Diameter (nm)
SDFT	65 ± 1.7	3.8 ± 0.7	84 ± 7	169 ± 19
DDFT	62 ± 1.4	5.7 ± 1.8	91 ± 3	215 ± 32 *
SL	68 ± 0.7 *	4.8 ± 0.6	74 ± 5	122 ± 14 *
CDET	57 ± 0.6 *	0.4 ± 0.2 *	88 ± 6	229 ± 36 *

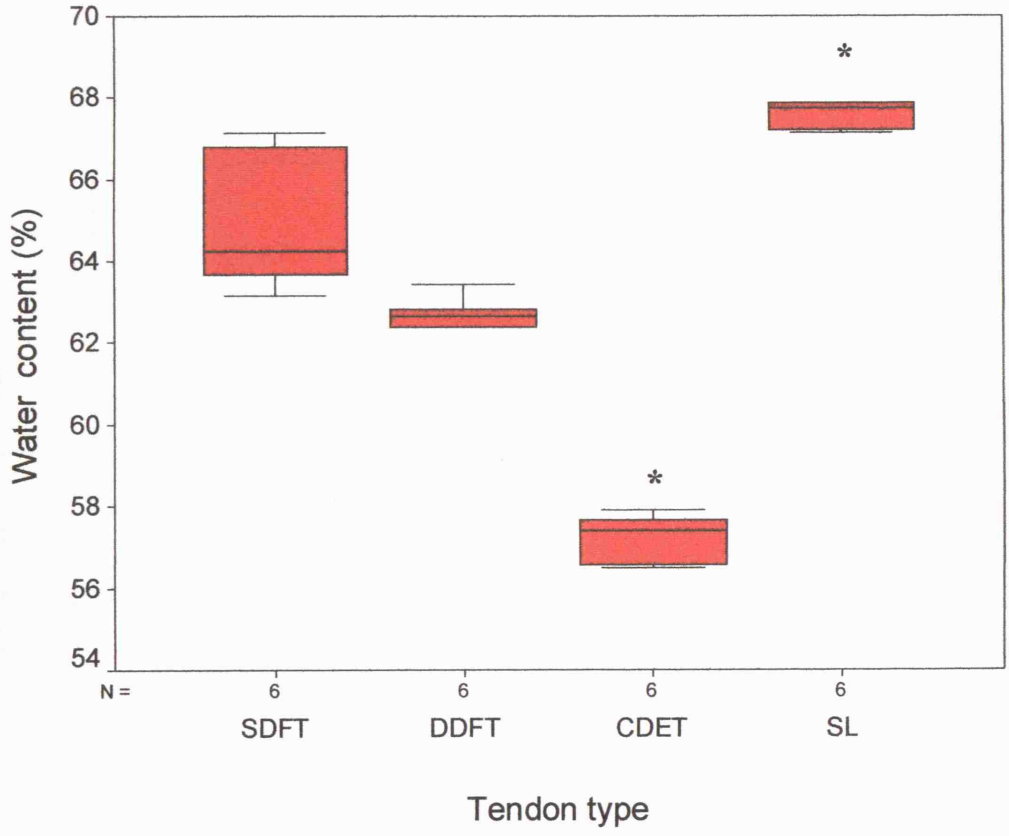


Figure 5.10: Boxplot showing water content for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* Denotes a significant difference relative to the SDFT.

The GAG content was significantly different **(Figure 5.11)** between the different structures ($p \leq 0.001$). GAG content results showed that the mean value of the DDFT (5.7 ± 1.8 μg/mg) was the highest, followed by the SL (4.8 ± 0.6 μg/mg), SDFT (3.8 ± 0.7 μg/mg), and the CDET (0.4 ± 0.2 μg/mg) was the lowest **(Table 5.2)**. The GAG content of the SDFT was significantly higher than the CDET ($p \leq 0.001$) but not significantly different from the DDFT or the SL **(Figure 5.11, Table 5.2)**.

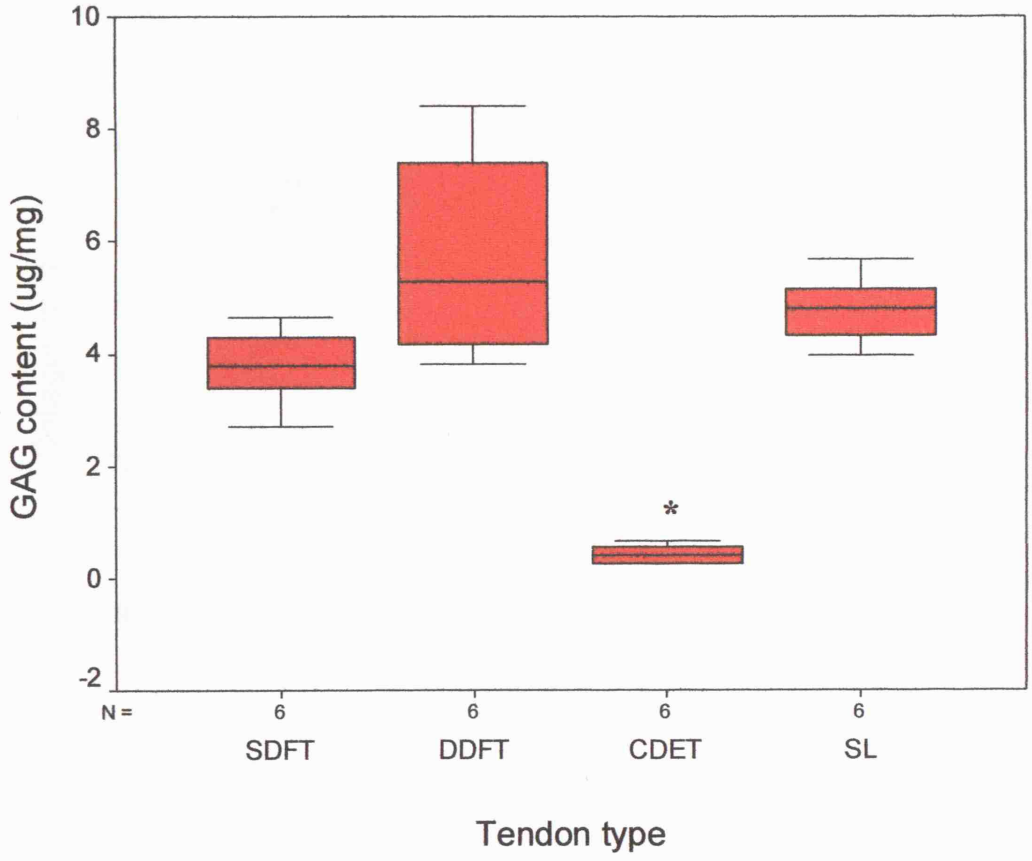


Figure 5.11: Boxplot showing GAG content for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* Denotes a significant difference relative to the SDFT.

The collagen content (expressed as a percentage of the dry weight of the tendon tissue) was not significantly different **(Figure 5.12)** between the different structures. Collagen content results showed that the mean value of the DDFT (91 ± 3 %) was the highest followed by the CDET (88 ± 6 %), SDFT (84 ± 7 %) and the SL (74 ± 5 %) was the lowest **(Table 5.2)**. The collagen content of the SL was significantly lower than the DDFT ($p \leq 0.001$) and the CDET ($p=0.007$) **(Figure 5.12)**.

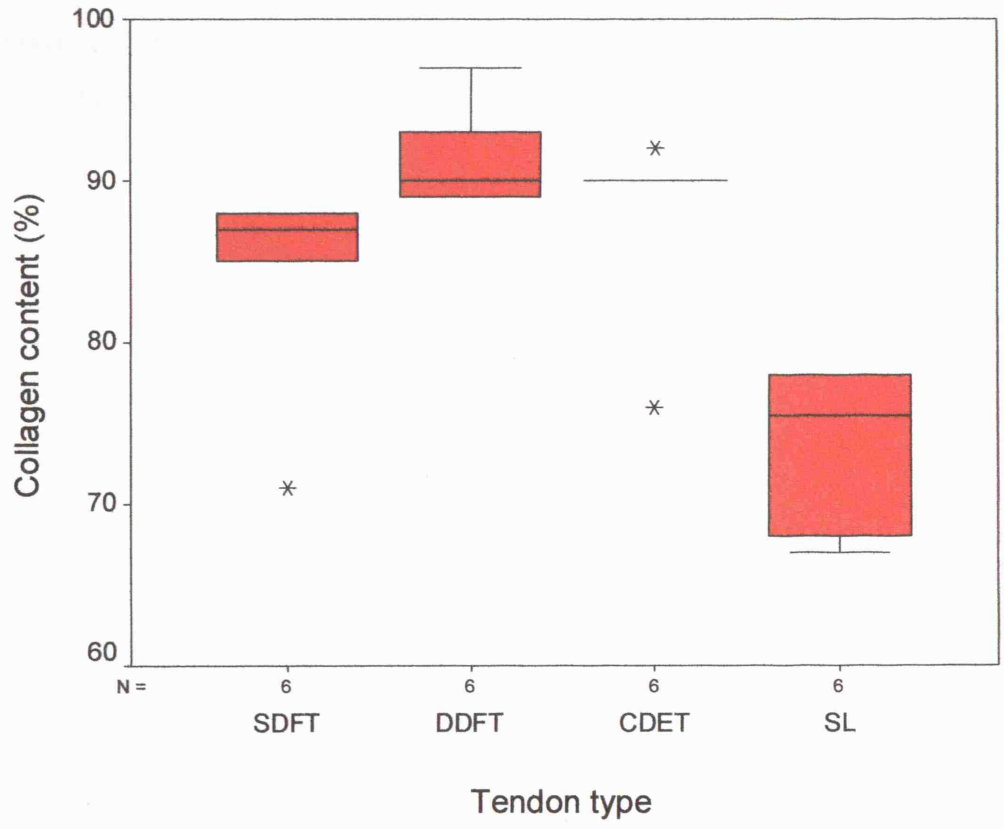


Figure 5.12: Boxplot showing collagen content for the SDFT, DDFT, CDET and SL. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* represents an extreme value.

5.5.5 Relationship Between Mechanical Properties, Morphology and Matrix Composition

There was a significant positive correlation between MAD and elastic modulus ($p=0.006$) in the SDFT, DDFT, CDET and SL **(Figure 5.13)**. Tendons with a higher elastic modulus tended to have larger fibril diameters. The water content showed a significant negative correlation ($p\leq0.001$) with elastic modulus for the different structures **(Figure 5.14)**. Tendons with a higher elastic modulus tended to have a lower water content. The GAG content showed a significant positive correlation ($p\leq0.001$) with the water content for the different structures **(Figure 5.15)**. Tendons with a higher water content tended to have a higher GAG content.

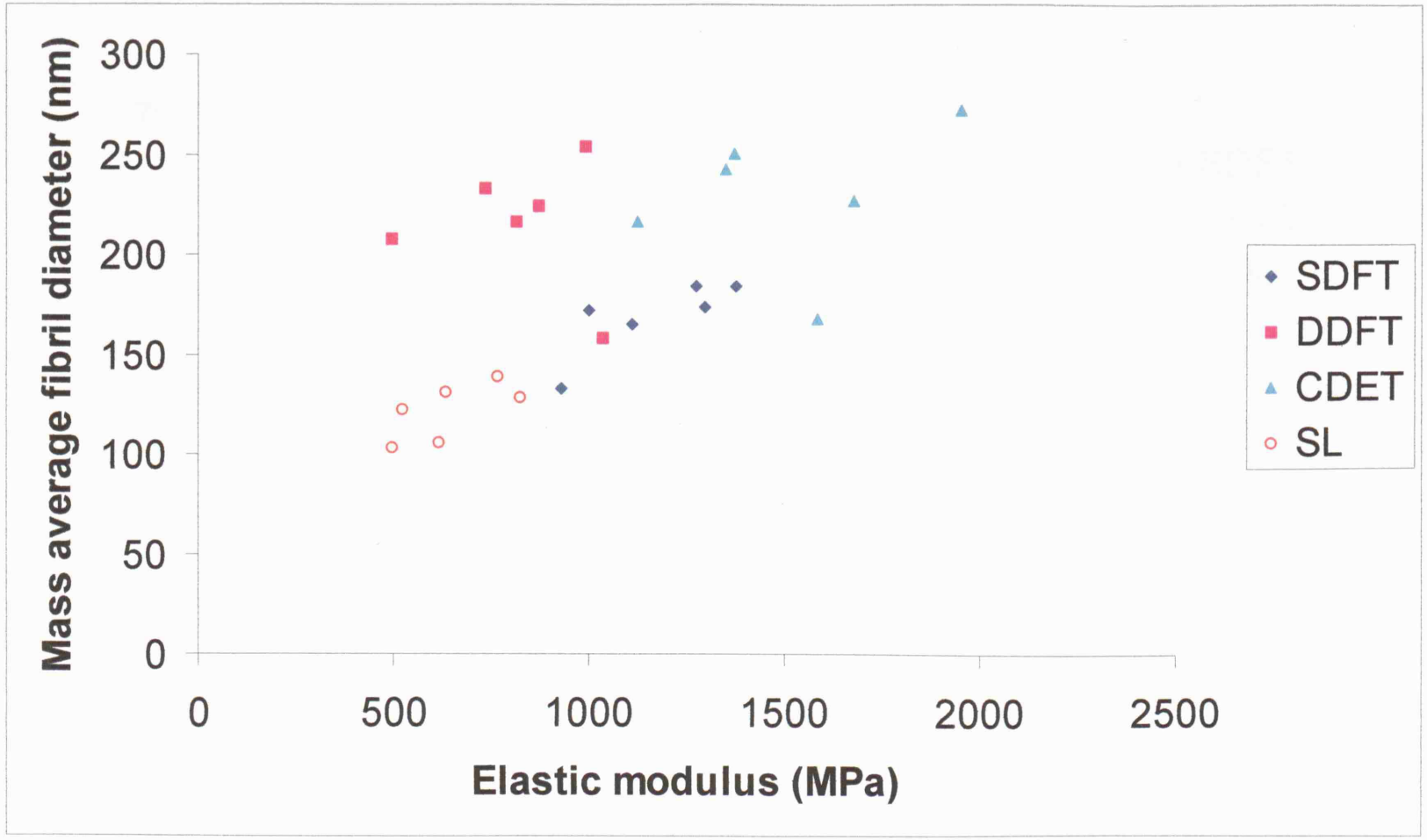


Figure 5.13: Relationship between mass average fibril diameter and elastic modulus in the SDFT, DDFT, SL and CDET. (R^2=0.3194, n=6, p=0.006).

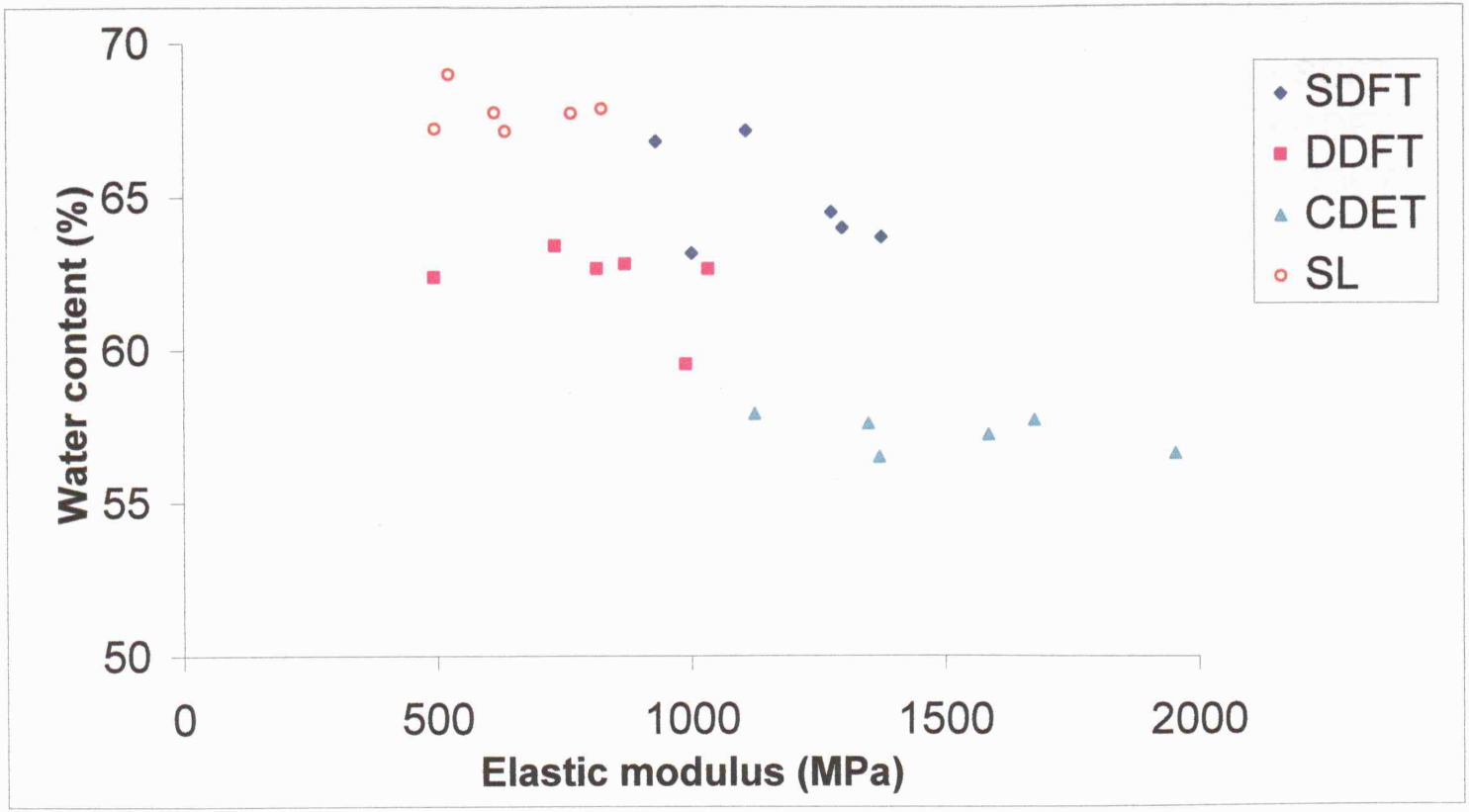


Figure 5.14: Relationship between water content and elastic modulus in the SDFT, DDFT, SL and CDET. (R^2= 0.5327, n=6, p<0.001).

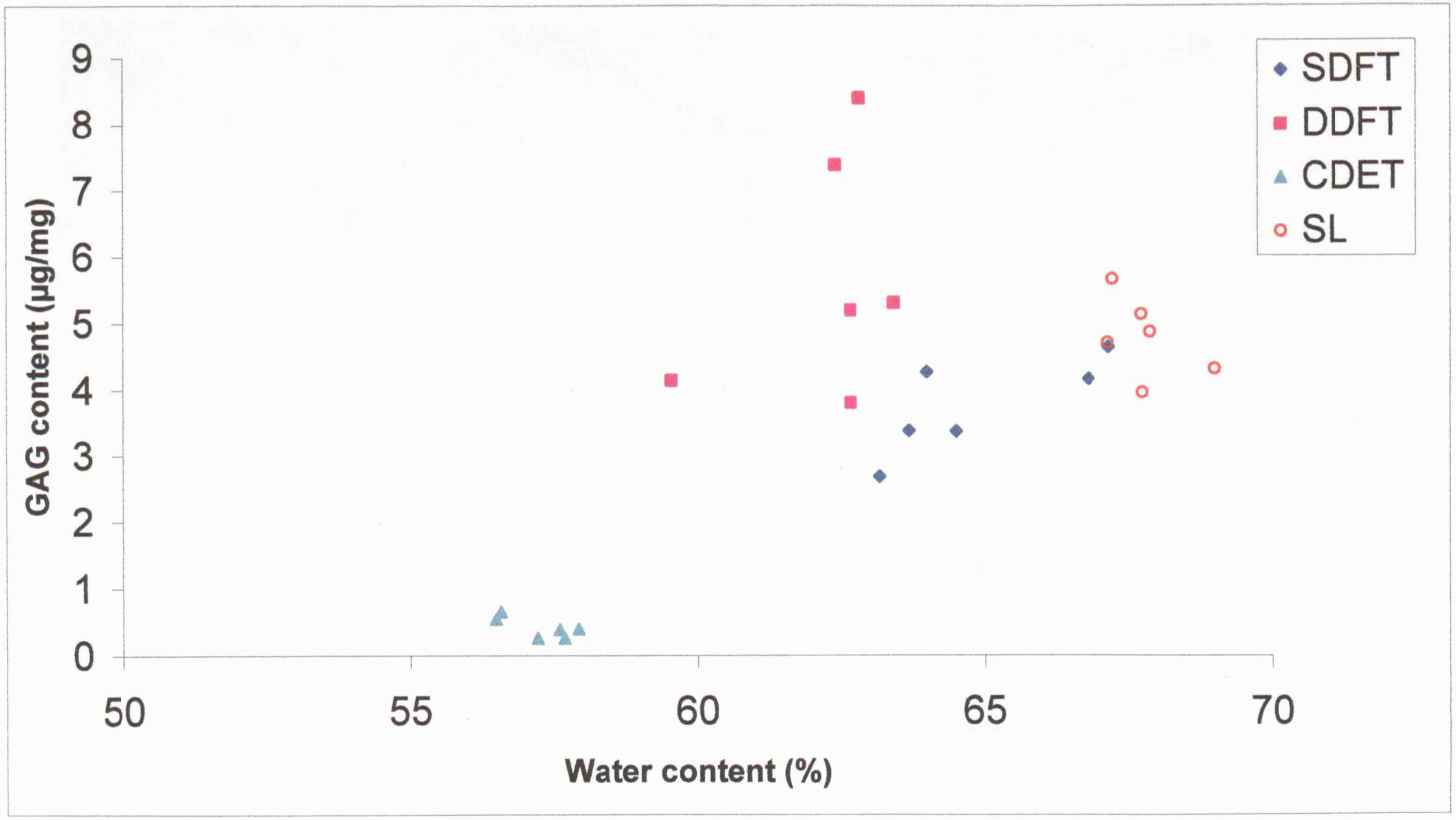
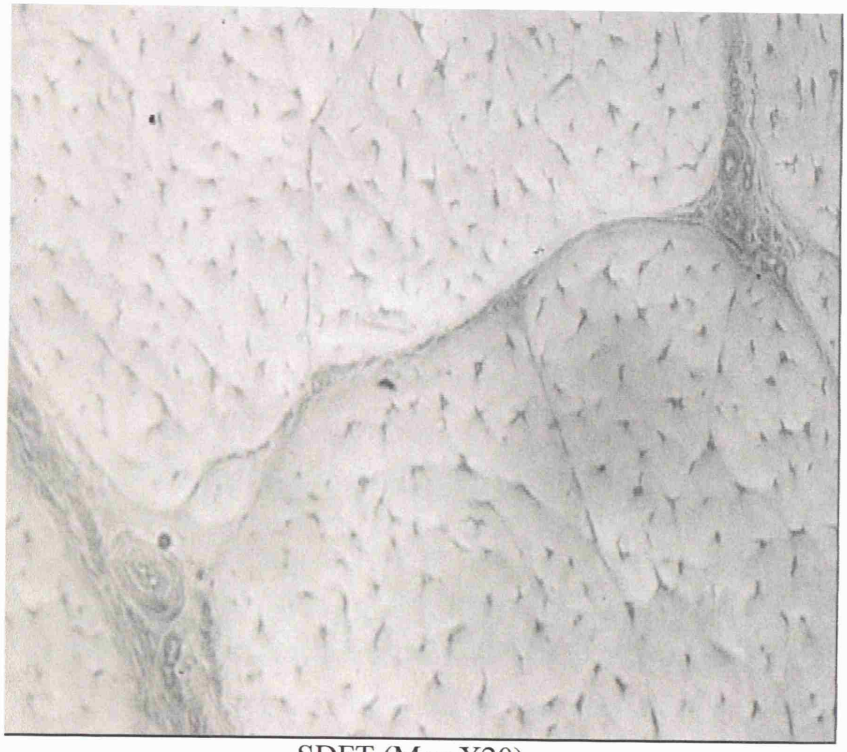
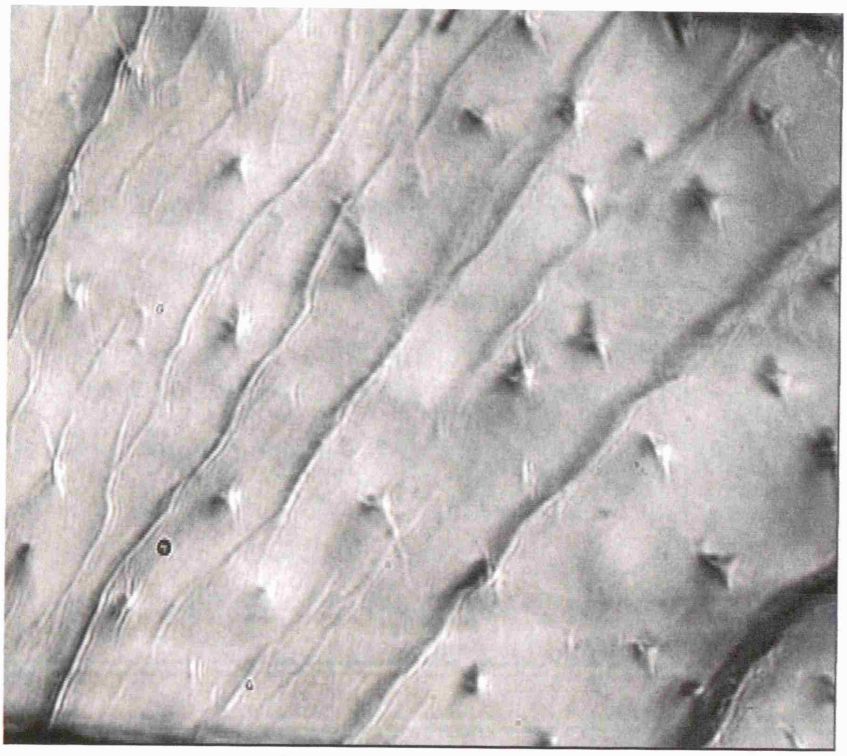


Figure 5.15: Relationship between GAG content and water content in the SDFT, DDFT, SL and CDET. (R^2= 0.4555, n=6, p=0.005).

5.5.6 Fascicle Morphology



SDFT (Mag X20)



CDET (Mag X40)

Figure 5.16: Resin embedded transverse sections showing fascicle morphology of SDFT and CDET.

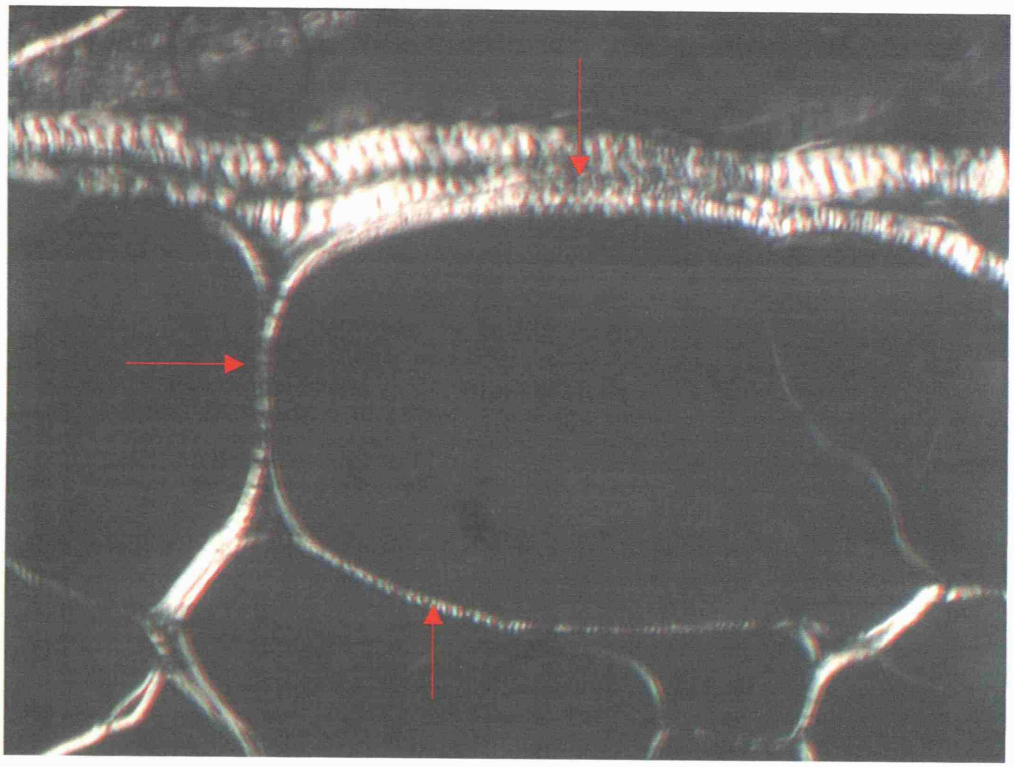


Figure 5.17: Resin embedded transverse section showing fascicle morphology in the SDFT under polarised light microscopy (Mag X4). Arrows indicate the presence of crimp. Top arrow indicates crimp in epitenon.

Qualitative assessment of fascicle morphology was made from the resin histology transverse sections. It was interesting to note that the morphology in the SDFT and CDET was different in that the fascicles varied in size and cross-sectional shape. The shape of the fascicles did not appear to be regular and fascicles in the SDFT were more rounded in shape whereas the fascicles of the CDET were 'finger like' in appearance **(Figure 5.16)**. In the SDFT, blood vessels were present at the edges of adjacent fascicles and the presence of interfascicular septa was evident. The distribution and density of tenocytes was also different between tendons. Many tenocytes could be seen on both the SDFT and the CDET, although the SDFT appeared to have more tenocytes present **(Figure 5.16)**. In the SDFT, the presence of crimp could be seen between fascicles when sections were viewed under polarised light **(Figure 5.17)**.

5.6 DISCUSSION

5.6.1 Material properties of specific tendons

The results of this study demonstrate that the collagenous structures within the equine distal forelimb have different structural and material properties. The CDET has the smallest CSA and as expected fails at the lowest force. The CDET has the highest elastic modulus and ultimate stress so is therefore composed of a stiffer material. In comparison, the SL has the largest CSA and as expected fails at the highest force. The SL has the lowest elastic modulus and ultimate stress so is therefore composed of a much less stiff material. The elastic modulus of 1510 MPa and ultimate stress of 189 MPa in the CDET are similar to values obtained by Batson (2002). The elastic modulus of 643 MPa and ultimate stress of 91 MPa in the SL are similar to values obtained for other *in vitro* studies (Jansen & Savelberg, 1994; Riemersma & Schamhardt, 1985). The stress-strain curves obtained in the present study are in agreement with previous descriptions (Abrahams, 1967; Herrick *et al.*, 1978; Ker, 1981; Woo *et al.*, 1981).

Our results show that different structures are also composed of tissue with different material properties. Tendons with a higher MAD had a higher elastic modulus, indicating a stiffer tendon matrix, which may increase its resistance to injury. The CDET was composed of a stiffer material and had larger diameter collagen fibrils whereas the SL was composed of a less stiff material and had smaller diameter collagen fibrils. The DDFT did not seem to fit the trendline as well as the other structures **(Figure 5.13)**. It appears that in the DDFT the size of the collagen fibrils are larger than expected for the elastic modulus, indicating that this may not be a simple relationship between fibril diameters and elastic modulus. Other factors may influence the elastic modulus other than the MAD such as tendon composition factors, for example, fascicle size, water content, collagen content or GAG content. It appears that the DDFT may be showing characteristics of a non-energy storing tendon and is also acting to position the leg in flexion. If the DDFT is in fact a non-energy storing tendon this may explain why this flexor tendon has a very low incidence of injury compared to the energy storing SDFT and SL.

Flexor tendons are under high stress during locomotion. The elastic modulus of 823 MPa and ultimate stress of 100 MPa in the DDFT are similar to values obtained for other *in vitro* studies (Jansen & Savelberg, 1994; Riemersma & Schamhardt, 1985). The mechanical properties of equine SDFTs are similar to those of other mammalian tendons. The elastic modulus of 1165 ± 178 MPa and ultimate stress of 131 ± 27 MPa in the SDFT are similar to values obtained in other studies (Batson, 2002; Jansen & Savelberg, 1994; Riemersma & Schamhardt, 1985). These values are also similar to those found in tendons from the deer, wallaby and donkey (Bennett *et al.*, 1986), the plantaris and gastrocnemius of the sheep (Ker, 1981), and the camel (Alexander *et al.*, 1982). The SDFT is subjected to very high loads of 10-20 kN (Goodship *et al.*, 1994) and stretches up to 16 % *in vivo* during high speed locomotion (Stephens *et al.*, 1989). This is very close to its failure strain *in vitro* which has been reported to be between 12 – 20 % (Goodship *et al.*, 1994). This tendon appears to work near its safety limit to function as an elastic energy store and is therefore more vulnerable to being injured. This may explain the high incidence of partial rupture of this specific tendon.

5.6.2 Limitations of mechanical testing in the present study

Mechanical testing of tendon *in vitro* can introduce several potential sources of errror. Typically, tensile tests involve gripping the tendon specimen, either directly at both ends using clamps or indirectly only at one end by gripping the bones to which the tendon is attached, and pulling it in a precise and well-controlled fashion until rupture of the tendon occurs. Previous studies have used ordinary (non-freezing) clamps which have been of a wide variety of designs, and used on different sized tendons (Abrahams, 1967; Benedict *et al.*, 1968; Ker, 1981; Ker *et al.*, 1986; Shadwick, 1990; Wang & Ker, 1995; Woo *et al.*, 1981). In the majority of mechanical testing protocols tendon specimens are clamped using specially designed freezing, hydraulic and pneumatic clamps with roughened gripping surfaces. Clamping of tendon tissue for mechanical testing can be a difficult task as slippage from the clamp is a problem commonly encountered. The tendon specimen needs to be held into the clamp rigidly at high loads without causing damage to it. Freezing the specimen in with liquid CO_2 seemed to successfully alleviate this problem because the frozen part of the tendon is not easily damaged by compressive forces

and it can be gripped tightly in the jaws of the clamp while the area of tendon outside the clamps can remain at room temperature (Riemersma & Schamhardt, 1982). Another potential problem is that once a specimen is frozen into the clamps this can cause a stress concentration in the region of the clamp, resulting in premature failure of the tendon at the freeze line. Therefore, ultimate properties such as ultimate tensile stress and ultimate strain can only be determined if failure occurs between freeze lines on the tendon. In the present study, 15 of the tendons broke at the clamps and 9 of the tendons broke in the mid-metacarpal region of the tendon. Therefore, the values obtained for ultimate properties (i.e. ultimate stress and ultimate strain) will be slightly underestimated in those tendons which broke at the clamps. Tendon clamps were tightened to the same torque of 27 Nm for every tendon with a torque wrench prior to testing so that all tendons were tested in the same way and this successfully avoided tendons pulling out during mechanical testing.

In the present study the SDFT, DDFT, and SL tendons were pre-loaded to 100 N. The CDET was pre-loaded to 25 N because this tendon is approximately ¼ the CSA of the other tendons. A small pre-load was required to enable the materials testing machine to be set to load control. These values were chosen because they were small but reproducible so that all tendons were mechanically tested in the same manner. This corresponds to 1% predicted load of failure, which is a negligible amount. For this reason, the stress-strain curves obtained have a small portion at the beginning of the 'toe region' missing. Other authors (Crevier *et al.*, 1996; Ker *et al.*, 2000) have also used pre-loading in mechanical testing protocols.

Frozen storage does not cause the mechanical properties of tendon to be different from those samples tested fresh. Ker (1981) found no perceptible difference in elastic properties between sheep plantaris tendons that were tested fresh and ones that had been stored frozen at –20 °C. Woo *et al.* (1986) showed that the biomechanical properties of ligaments and tendons after prolonged freezing storage were not different from fresh samples. It has also been shown that the mechanical properties of equine tendons do not differ between fresh and frozen specimens (Birch, unpublished data). In our study, mechanical testing was carried out on fresh tissue that was used within 24 hours of death. Due to the fact that tendons contain a large amount of water (60-80%), their mechanical properties can be affected by

dehydration. It is important during testing to ensure that tendons are not allowed to dry out. The tendons were therefore wrapped in clingfilm between dissection and mechanical testing to avoid this.

Tendon is a viscoelastic material and changes in temperature will therefore affect its mechanical properties. An increase in temperature will lead to a decreased elastic modulus and an increase in strain at the same stress (Alexander, 1988). Therefore, the influence of environmental temperature on the biomechanical behaviour of tendons should be considered. All mechanical testing in our study was performed at room temperature (20°C ± 2°C) so the effect of temperature on our results would likely to have been small.

There were other mechanical properties which were not measured in the present study. These included toe region properties, yield point, and energy to failure. These could be included in future studies on mechanical properties of tendons.

5.6.3 Structural properties of specific tendons

The variation in the mechanical properties may be achieved by variation in the material properties and the size of the structure. The measurements obtained in this study for CSA of 100 mm^2 for the SDFT, 167 mm^2 for the DDFT, 192 mm^2 for the SL, and 30 mm^2 for the CDET were similar to those reported by Birch *et al.* (1999a). Tendons with a larger CSA are stronger and stiffer as a structure. This could be seen particularly in the DDFT and SL, which resulted in them having a larger CSA and being stronger tendons, although the material properties indicated that they were composed of a less stiff material. However, the results from the present study are not in agreement with the work of Ker (1999) who illustrated that structural design determines the tendon's CSA relative to that of its muscle and, hence, the maximum stress to which the tendon may be subjected to in life. Stress-in-life is defined by Ker (1999) as the stress in a tendon when its muscle is exerting maximum isometric stress. Stress-in-life varies widely between tendons. The length of a tendon is determined by its position in the animal's body. The only structural design variable is thickness. According to Ker (1999), the thinner the tendon, the more it will stretch under a given force from its muscle. Ker (1999) concluded that tendons are all made

of the same material with tendon thickness being the controlled variable, and that structural thickness is achieved by changing the thickness of the tendon rather than the material stiffness. In contrast, our study has shown that tendons with different functions are made of material with different mechanical properties, and that the material of tendons with different functions is also different in terms of collagen fibril morphology, and matrix composition which is not in agreement with the work of Ker (1999).

There was one outlier in terms of tendon CSA in the present study. This horse was a Thoroughbred cross that was a 17.1 hh, 7 year old gelding that had been used for general purpose riding, hunting, dressage and jumping. This horse had been destroyed for temperament problems. The CSA of the left SL was 278 mm^2, which was much higher than the other SL values. This horse may have had a SL injury, which is why the left tendon appeared larger. It would have been expected that this horse would have had a lower MAD value indicating a higher proportion of small fibrils, which may have been due to disagregation of large fibrils. However, the collagen fibril morphology was not affected for this horse.

5.6.4 Collagen organisation in specific tendons

The collagen component and the way in which it is organised is responsible for the mechanical properties of a tendon and therefore differences in the arrangement of collagen are evident in tendons with different functions. Tendons with different functions have a specific material stiffness, which correlates with the collagen fibril diameters. Tendons which are composed of a less stiff material (i.e. low elastic modulus) and are therefore more easily stretched, have a similar collagen content to tendons made of a stiffer material but have smaller diameter collagen fibrils. Those tendons with larger collagen fibril diameters also have a lower GAG content, which may be responsible for preventing further lateral growth of the fibrils. Tendons with smaller diameter collagen fibrils also have a higher water content and it may be possible to detect this using imaging techniques, which would allow a prediction of tendon strength and stiffness to be made *in vivo*.

MAD values obtained in the present study of 169 nm in the SDFT, 215 nm in the DDFT, 122 nm in the SL and 229 nm in the CDET were all approximately 10-20 nm higher than those of Patterson-Kane *et al.* (1997a, 1998). This difference may be due to the fact that the tissue processing technique had been modified and also a different image analysis system was used for measurement of collagen fibrils. When photographing collagen fibrils on the TEM, the collagen fibrils needed to be circular in appearance because if they were elliptical in shape this would underestimate the collagen fibril diameter measurements because an elliptical shaped fibril would measure less in diameter than a circular fibril. Therefore, only electron micrographs with circular fibrils were used in this study. If any fibrils were slightly oblique, then the smallest diameter of the fibril was used. This problem usually occurred due to incorrect sectioning angle or embedding the tissue in the wrong orientation within the block. Our results are in agreement with Parry *et al.* (1978b), who has previously shown that small fibrils are the most important for providing the tendon with elasticity although they are mechanically weaker than the large ones.

In the present study there were two outliers in terms of MAD values. The first horse was an Irish show horse that was a 17hh, 7 year old gelding who had been destroyed for behaviour problems. This horse had the lowest MAD (134 nm) for the SDFT from the group. Interestingly, this horse also had the lowest elastic modulus (931 MPa) and ultimate stress (92 MPa). In comparison, the CSA from both the left (125 mm^2) and right (124 mm^2) SDFTs of this horse were the highest from the group of six horses. Therefore, these tendons appeared to be slightly larger than the other SDFTs and this may have been the beginning of the development of tendon injury. The fact that the material properties were also lower indicates that the tendon may have been compensating if enlarged by having a lower elastic modulus and this may have increased the size of the structure. The second horse that was an outlier in terms of MAD, was a 15.1 hh, 11 year old Thoroughbred mare which died from small intestinal disease. The mare had a 1 day old premature foal, who was septic. This horse had the lowest MAD (158 nm) for the DDFT from the group. Interestingly, this horse had the lowest tendon CSA (123 mm^2), the highest elastic modulus (1034 MPa) and the highest ultimate stress (134 MPa) for the group. These results appear to be the opposite way round from that of the first horse.

Results from the present study show that the collagen fibril diameter distributions were bimodal for the SDFT, DDFT, SL and CDET. The CDET was the tendon that came the closest to having a unimodal distribution. These findings are in agreement with Parry *et al.* (1978b) who reported that the SDFT, DDFT, SL, and CDET have a bimodal distribution from maturity to senescence. The collagen fibril diameter distribution is related to the mechanical properties of tendon. Parry & Craig (1988) illustrated that the creep-inhibition property of a tissue is directly related to the percentage of small-diameter fibrils present whereas the ability of the tissue to withstand high stress levels is related to the percentage of large-diameter fibrils in the tissue. It is the larger diameter fibrils that are believed to have a particularly important role in sustaining the tensile load resistance of the tissue.

Qualitative assessment of fascicle morphology in the present study showed that the morphology of the SDFT and CDET was different in that the fascicles varied in size and cross-sectional shape. The distribution and density of tenocytes was also different between these tendons. Interestingly, the presence of crimp could be identified between adjacent fascicles in the SDFT, which may provide a role to maintain the mechanical integrity of the fascicles so that their shape is not altered too much by a sliding mechanism as a tendon is stretched, which would then have an effect on water content, water and cell pressures, and tenocytes. Further work is however required to determine what is happening to the crimp in transverse section. Goodship & Birch (2001) have also demonstrated that on histological section there are differences in the morphology and distribution of tenocytes in different tendons. However, little is known about the factors that relate to the presence of particular cells within specific tendons in terms of their functional roles. There is evidence that tenocytes respond to mechanical stimulation in cell culture. Studies have shown that tenocytes can synthesise small peptides that may regulate both proliferation of cells and synthesis of extracellular matrix components (Banes *et al.*, 1999). Cells present in the inter-fascicular septa have been shown to play a role in the synthesis of growth factors that may influence the metabolic activity of the fibrillar tenocytes (Cauvin *et al.*, 1998). The fibrillar tenocytes themselves are varied in morphological appearance and in distribution as a function of specific tendon and age (Goodship *et al.*, 1994; Smith & Webbon, 1996; Webbon, 1978). These cells are thought to be responsible for the production and maintenance of the surrounding extracellular

matrix (Goodship *et al.*, 1994). Unfortunately in the present study, it was not possible to obtain quantitative fascicle morphology data due to problems encountered with our experimental technique. An attempt was made to measure the fascicle CSA of the SDFT, DDFT, SL and CDET using resin histology, but in the end this was only possible in the SDFT because the fascicles could be correctly distinguished in this tendon. However, because of the problem encountered in these histology sections with large cracks through them, which appeared to be gaps where the resin had pulled the fascicles apart along the septa, it was decided to measure fascicle CSA in the SDFT by a SEM technique developed to obtain quantitative fascicle morphology data (see Chapter 3 & 6).

Horse age, breed and exercise level are known to have an influence on the mechanical properties of tendon (Birch *et al.*, 1999a; Crevier *et al.*, 1996; Crevier-Denoix *et al.*, 1998; Gillis *et al.*, 1995; Kasashima *et al.*, 2002b). The group of six horses used in this study were within the age range of 4-12 years and were a mix of Thoroughbred, Thoroughbred-type and non-Thoroughbred adult horses which all had varying levels of exercise **(Table 3.1, Chapter 3)**. This may contribute to any variations present in material properties or morphology of the tendons. However, as the sample size in the present study was small (n=6), it was not possible to statistically analyse differences of particular horse age, breed or level of exercise. It was also not possible to statistically analyse age related changes to mechanical properties, morphology and matrix composition in this group of six horses. The subgroup of six horses was chosen to be most representative of the whole group of thirty horses. However, it was more important for this part of the study that the horses were of a close age range (i.e. 4-12 years) so that relationships between mechanical properties, morphology, and matrix composition in tendons with different functions could be assessed without other factors influencing the relationships such as horse age. A further study could be carried out in the future to look at the effects of specific ages, horse breeds and exercise levels using a larger group of horses.

5.7 CONCLUSIONS

- The SDFT, DDFT, SL and CDET have evolved for specific functions, which are reflected in their material properties and collagen fibril morphology. Structural properties, material properties, collagen fibril morphology and matrix composition are significantly different between the SDFT, DDFT, SL and CDET.

- Collagen fibril morphology correlates with material properties in terms of mass average fibril diameter and elastic modulus. Energy storing tendons that function effectively as biological springs have a lower elastic modulus (i.e. have a less stiff matrix) and are composed of smaller diameter collagen fibrils than non-energy storing positional tendons.

- Tendons which are composed of a less stiff material, and are therefore more easily stretched, have a higher water content and higher GAG content than tendons made of a stiffer material but have smaller diameter collagen fibrils.

- These studies enable the relationships between the structure and mechanical properties of tendon to be determined, from which the long-term aim is to allow identification of horses most at risk from tendon injury.

CHAPTER 6

DO AGE RELATED CHANGES TO TENDON MORPHOLOGY ACCOUNT FOR CHANGES IN MECHANICAL PROPERTIES AND INCREASED INCIDENCE OF TENDON RUPTURE?

6.1 INTRODUCTION

Achilles tendon injury is a common orthopaedic problem in both athletes and non-athletes and the incidence of injury increases with age (Kannus, 1997; Maffulli, 1999). It is thought that tendon rupture usually occurs as a result of an accumulation of degenerative change within the central core of the tendon (Kannus & Jozsa, 1991). The horse also suffers a high incidence of tendon partial rupture, and the tendon most often involved is the SDFT. The incidence of injury also increases with age and shows a similar pathogenesis to that seen in the human Achilles tendon (Goodship & Birch, 2001). The horse is therefore an ideal model to study age related changes to tendons.

Previous studies have shown that ageing results in a decrease in mechanical properties in the human Achilles tendon (Birch *et al.*, 2001). A correlation between mechanical properties and collagen organisation in equine tendons has also been shown (refer to Chapter 4 & 5). This may account for the increased incidence of tendon injury in older age. An increased incidence of rupture in older age groups at a specific site suggests that age related changes occur to the tendon matrix at that site which weakens the matrix predisposing to rupture. Another possible explanation may be that an accumulation of damage is occurring due to exercise. The fibrous protein collagen is responsible for the tensile strength of the tendon, however previous studies have found no difference in the total collagen content in the central core of the SDFT between young and old horses, or as a consequence of exercise in the SDFT of both young and old horses (Birch *et al.*, 1999b). The morphological hierarchy of collagen organisation into fibrils and fascicles is very important to ensure mechanical integrity of tendon. Thus, age related changes in mechanical properties may occur as a result of changes in morphology rather than absolute amounts of collagen. To date no study has determined whether age related changes occur to both fascicle and fibril morphology in a single study, and whether these changes account for site specific tendon degeneration. Therefore, this will be the main focus of the present study.

fibrils, the units of tensile strength, residing in a hydrated glycoprotein-rich matrix (Kastelic *et al.*, 1978). Fascicles are surrounded by endotenon and do not have any direct attachments or communications with each other although they may interact through their surface zigzag waveform known as crimp (Kastelic *et al.*, 1978). Under the ordinary light microscope, small blood vessels and loose connective tissue can be seen between fascicles of collagen fibres and also between collagen fibrils. Fascicles vary in size and cross-sectional shape, and it is thought that the shape is influenced, but not determined by compression from the surrounding structures or by the presence of neighbouring fascicles (Kannus, 2000). Scanning electron microscopy (SEM) has been used successfully in the past to investigate the structure of fascicles in rat tail tendon (Rowe, 1985). Therefore, SEM will be used in the present study to investigate the structure of fascicles in the SDFT.

Several studies have attempted to investigate the relationship between tendon structure and function (Bay *et al.*, 1993; Derwin & Soslowsky, 1999; Haut *et al.*, 1992; Parry *et al.*, 1978b). Tendon extracellular matrix composition and structural organisation reflect the functional requirements of the tissue, although collagen fibrils have been the primary focus of previous structure-function relationships (Davankar *et al.*, 1996; Parry *et al.*, 1978a). Large diameter fibrils have a high density of intermolecular cross-links by virtue of their low surface area per unit volume ratio. They are thought to be stronger than small diameter fibrils which have a large surface area per unit volume, permitting greater interaction with interfibrillar matrix components which reduces slippage between fibrils and provides elasticity (Parry *et al.*, 1978a).

It has been suggested that pre-existing degenerative change may predispose the SDFT to subsequent mechanical failure (Stromberg & Tufvesson, 1969). The investigation of relationships between extracellular matrix structure and tissue mechanical properties in tendon has previously been studied by Derwin & Soslowsky (1999). In their study, they used the mouse tail tendon fascicle as an appropriate model to investigate these structure-function relationships quantitatively. The results demonstrated that fascicle stiffness and maximum load were positively and moderately correlated with mean collagen fibril

correlated with mean collagen fibril size. In another study, Bush (2002) investigated the mechanical properties of fascicles in the SDFT. They found that core region fascicles had a lower ultimate strain than the complete tendon, suggesting that as a tendon is stretched, the fascicles in the core region would fail before the complete tendon. This also suggests that movement occurs between fascicles. More recently, Screen *et al.* (2004) has also demonstrated that fibre sliding appears to be the major mechanism enabling tendon fascicle extension within the rat tail tendon.

6.2 Hypothesis

Ageing results in a decrease in elastic modulus (material stiffness) associated with site specific changes in tendon fascicle and fibril morphology in energy storing tendons.

6.3 Objectives

1. To determine whether age related changes occur in mechanical properties in the SDFT. In particular to determine whether ageing results in a decrease in elastic modulus.

2. To determine whether age related changes occur to tendon fascicle and fibril morphology. In particular, to determine whether tendon fascicle size and collagen fibril diameters decrease with age in the SDFT.

3. To determine whether age related changes to tendon morphology may account for site specific tendon degeneration, particularly to the central core of the SDFT.

6.4 Experimental Design

In this part of the study left forelimb SDFTs, which were mechanically tested to failure, will be used to assess whether age related changes occur to mechanical properties. SEM will be used to measure the CSA and septal width of fascicles

zones of the SDFT. The organisation of collagen in the matrix is important (refer to Chapter 1) and this will be assessed by measuring collagen fibril diameters in central and peripheral zones of the SDFT, using transmission electron microscopy (TEM). GAG concentrations in central and peripheral zones of the SDFT will also be measured since it is known that GAGs play a role in the development of collagen fibrils (Parry *et al.*, 1982). In addition water content and collagen content will be measured in central and peripheral zones of the SDFT to determine whether there are any differences in matrix composition between zones with increasing horse age.

6.5 RESULTS

6.5.1 Mechanical Properties

Elastic modulus decreased significantly (n=30, p=0.017) with increasing horse age in the SDFT **(Figure 6.1)**. This group of horses were of three horse types and elastic modulus in the Thoroughbred group decreased significantly with increasing horse age in the SDFT (n=14, p=0.043). Elastic modulus did not decrease significantly in the Thoroughbred type (n=8, p=0.232) and non-Thoroughbred group (n=8, p=0.468) with increasing horse age in the SDFT. Ultimate stress decreased significantly (n=30, p=0.01) with increasing horse age in the SDFT **(Figure 6.2)**. Ultimate stress decreased significantly in the Thoroughbred group (n=14, p=0.025) but did not decrease significantly in the Thoroughbred type (n=8, p=0.944) or in the non-Thoroughbred group (n=8, p=0.313) with increasing horse age in the SDFT. CSA of the whole tendon (98.5 ± 21.3 mm^2, n=30, p=0.079) did not change significantly with horse age **(Figure 6.3)**.

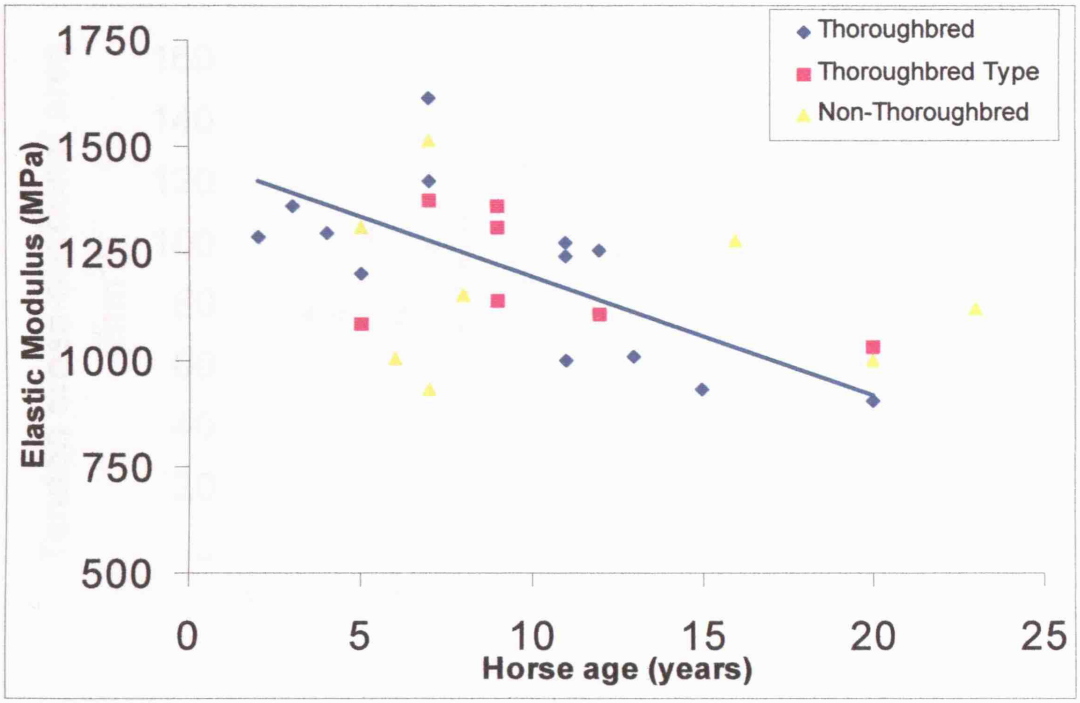


Figure 6.1: Elastic modulus in the SDFT against horse age.
(R^2=0.1882, n=30, p=0.017).

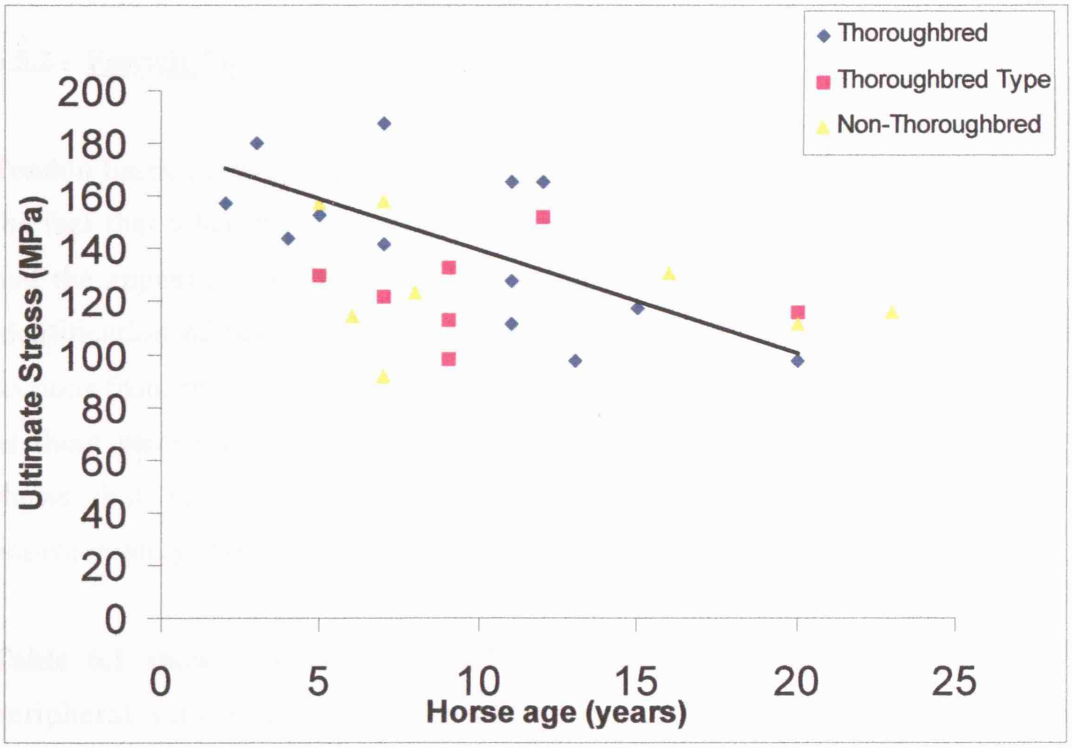


Figure 6.2: Ultimate Stress in the SDFT against horse age.
(R^2=0.2169, n=30, p= 0.01).

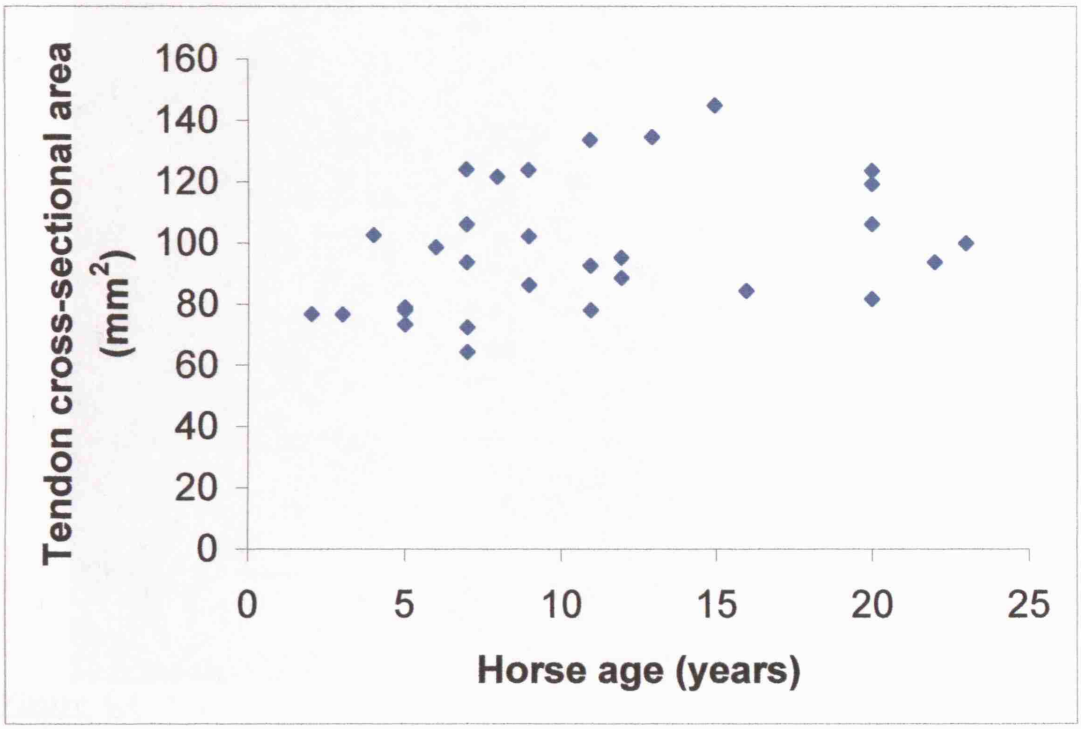


Figure 6.3: Tendon cross-sectional area against horse age.
(R^2=0.1064, n=30, p=0.079).

6.5.2 Fascicle Morphology

Tendon tissue needed to be snap frozen prior to preparation for the SEM due to the fact that when fresh tissue was lyophilised the samples viewed on the SEM had the appearance of holes throughout the specimen **(Figure 6.4)**. This made identification of fascicles difficult. Therefore, it was decided to only use the sections from the snap frozen specimens for quantitative fascicle measurements, as these were much better quality. **Figure 6.5** from snap frozen specimens, shows that fascicles within the medial peripheral zone of the SDFT are approximately 500 μm in diameter.

Table 6.1 shows fascicle CSA and septal width measurements for medial peripheral, central and lateral peripheral zones of the SDFT. Fascicles in the central zone of the SDFT **(Figure 6.6, Figure 6.8)** were significantly larger (n=30, p<0.001) than fascicles in the medial peripheral **(Figure 6.5, Figure 6.8)** and lateral peripheral zones **(Figure 6.7, Figure 6.8)**. Fascicle CSA was not

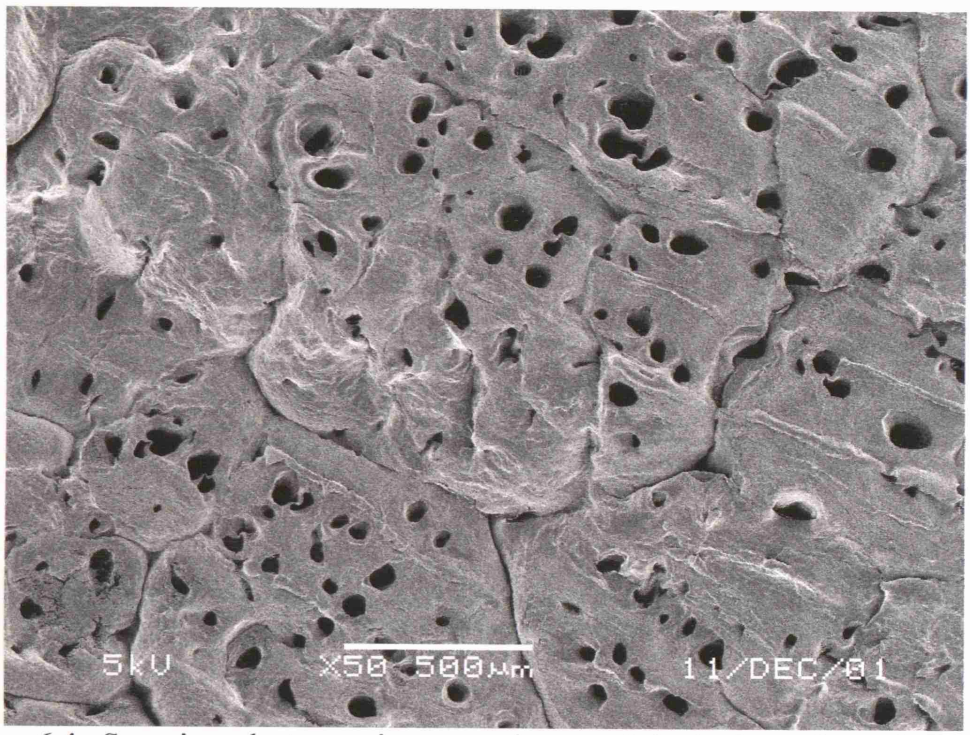


Figure 6.4: Scanning electron microscope image of fresh tissue from the SDFT showing the appearance of holes throughout the specimen.

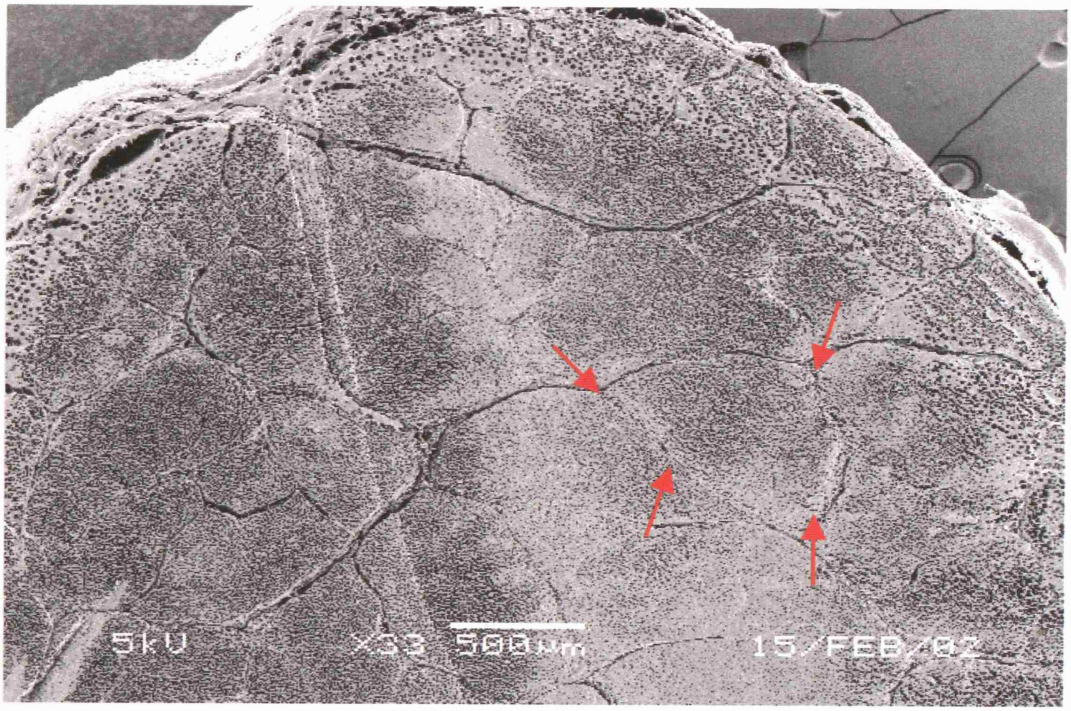


Figure 6.5: Scanning electron microscope image of the medial peripheral zone of the SDFT from a snap frozen specimen, showing fascicle morphology. (Arrows indicate a fascicle within this zone).

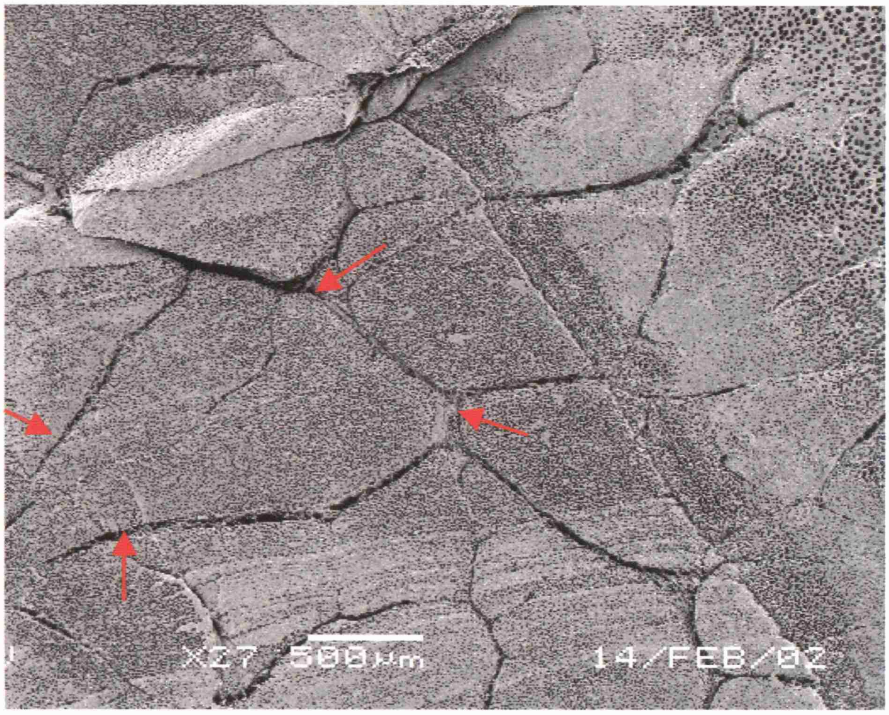


Figure 6.6: Scanning electron microscope image of the central zone of the SDFT showing fascicle morphology. (Arrows indicate a fascicle within this zone).

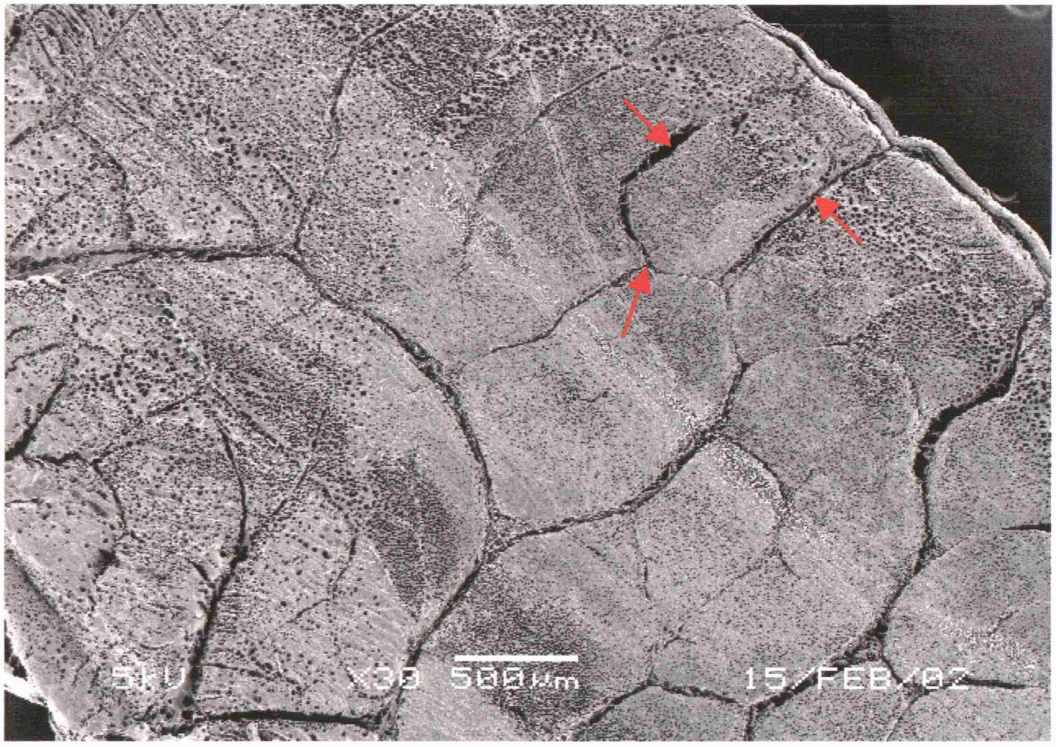


Figure 6.7: Scanning electron microscope image of the lateral peripheral zone of the SDFT showing fascicle morphology. (Arrows indicate a fascicle within this zone).

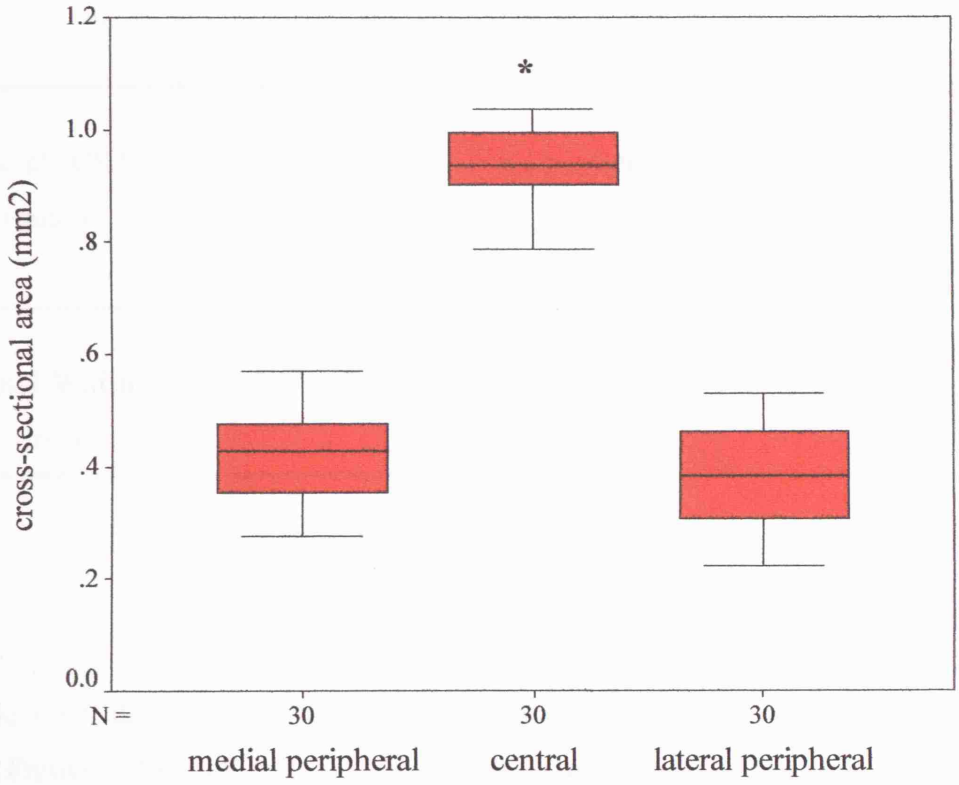


Figure 6.8: Box plot showing fascicle cross-sectional area values for different zones of the SDFT. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

* Denotes a significant difference relative to medial peripheral and lateral peripheral zones.

Table 6.1: Fascicle CSA (n=30) and septal width (n=24) measurements for the medial peripheral, central, and lateral peripheral zones of the SDFT. (* denotes a significant difference relative to medial peripheral and lateral peripheral zones).

	SDFT Medial Peripheral Zone	SDFT Central Zone	SDFT Lateral Peripheral Zone
Fascicle CSA (mm^2)	0.42 ± 0.08	0.94 ± 0.06 *	0.38 ± 0.09
Septal Width (μm)	15.67 ± 9.76	16.95 ± 9.4	14.95 ± 6.74

Fascicle CSA decreased significantly (p=0.002) with horse age in the central zone **(Figure 6.9)** but not in the medial peripheral (p=0.406) or lateral peripheral (p=0.804) zones.

Septal width measurements showed no significant difference between medial peripheral, central, and lateral peripheral zones **(Figure 6.10)**, and no correlation with horse age.

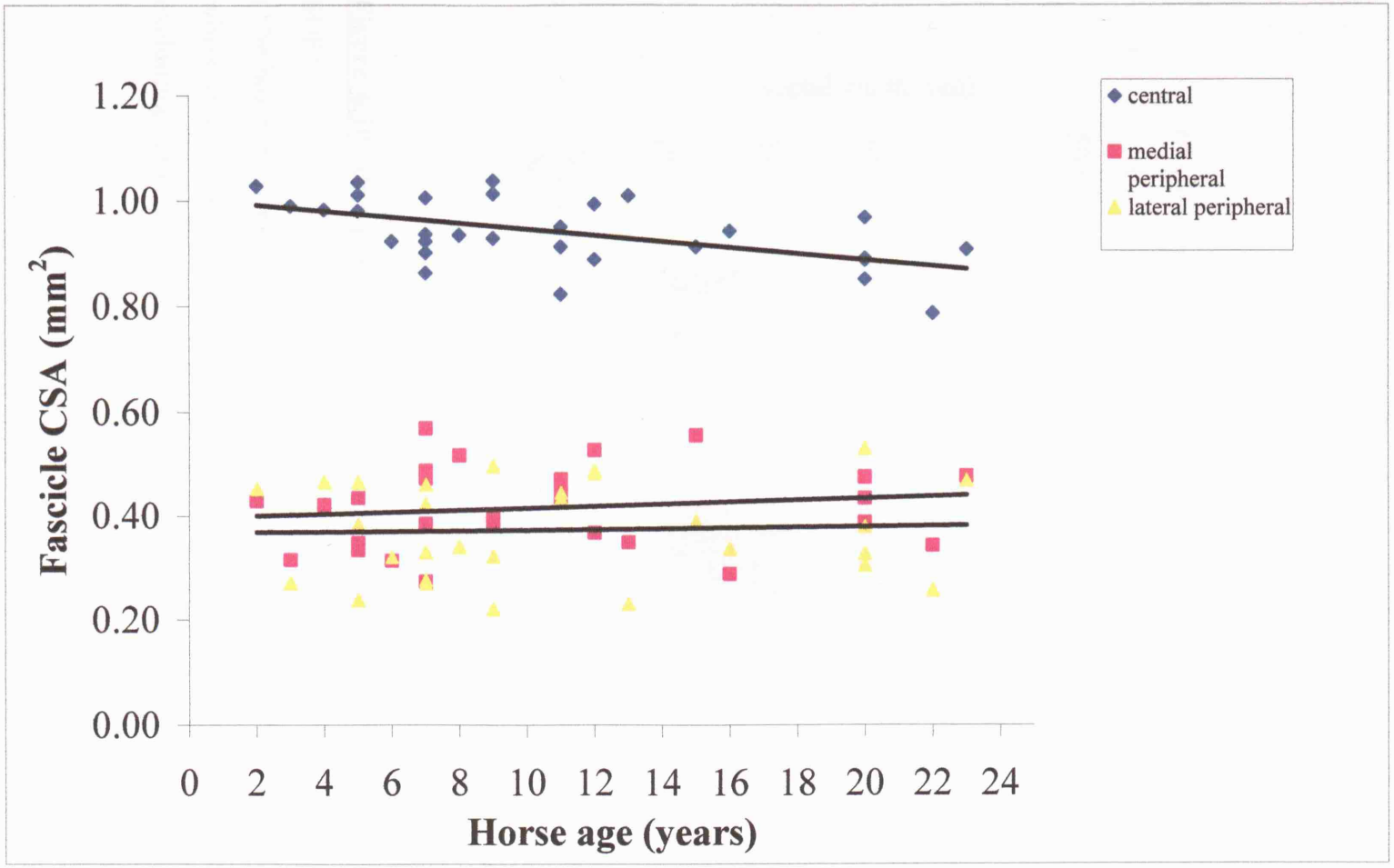


Figure 6.9: Fascicle cross-sectional area values in the central (R^2=0.2993, n=30, p=0.002), medial peripheral (R^2 =0.0248, n=30, p=0.406) and lateral peripheral (R^2=0.0022, n=30, p=0.804) zones of the SDFT against horse age.

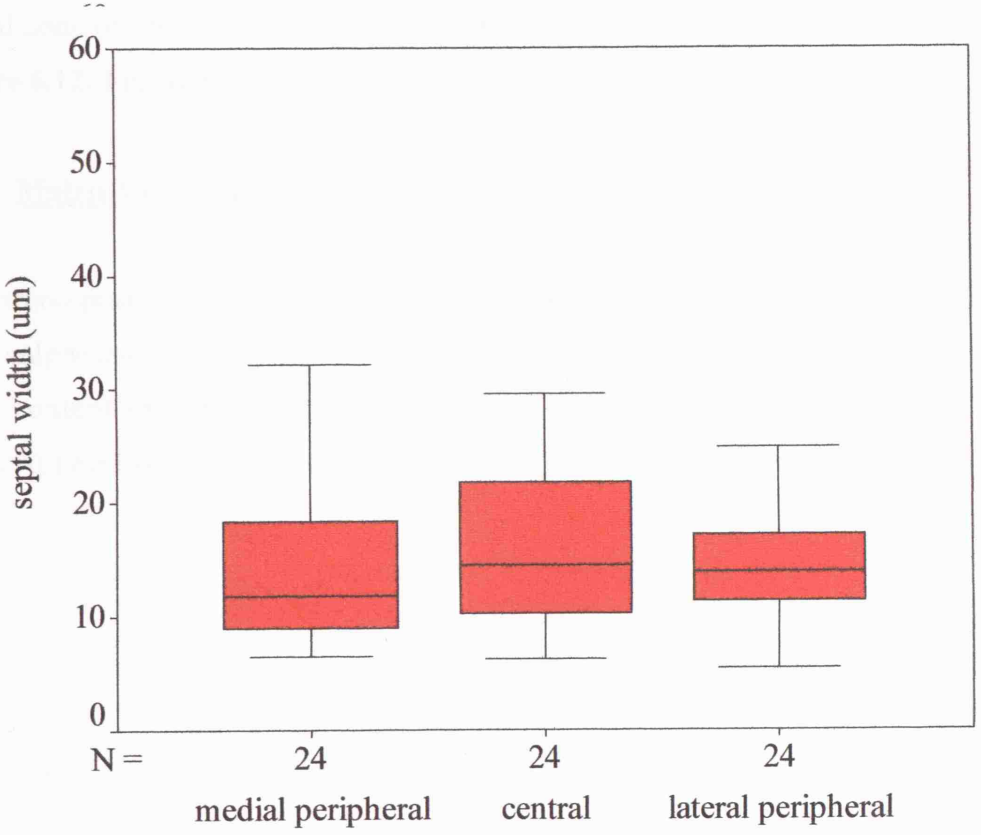


Figure 6.10: Box plot showing septal width values for different zones of the SDFT.

(The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).

6.5.3 Collagen Fibril Morphology

Collagen fibrils were compared in central and peripheral zones of the SDFT. Mass average collagen fibril diameters were not significantly different between central (185 ± 35.5 nm, n=7) and peripheral (178.4 ± 15.9 nm, n=7) zones of the SDFT **(Figure 6.11)** and the mass average collagen fibril diameters in the central zone of the SDFT decreased significantly (n=30, p=0.033) with horse age **(Figure 6.12; Figure 6.13)**.

6.5.4 Matrix Composition

Matrix composition was compared in central and peripheral zones of the SDFT. Total sulphated GAG content **(Figure 6.14)**, collagen content **(Figure 6.15)** and water content **(Figure 6.16)** showed no significant difference between central and peripheral zones of the SDFT **(Table 6.2)** and no correlation with horse age.

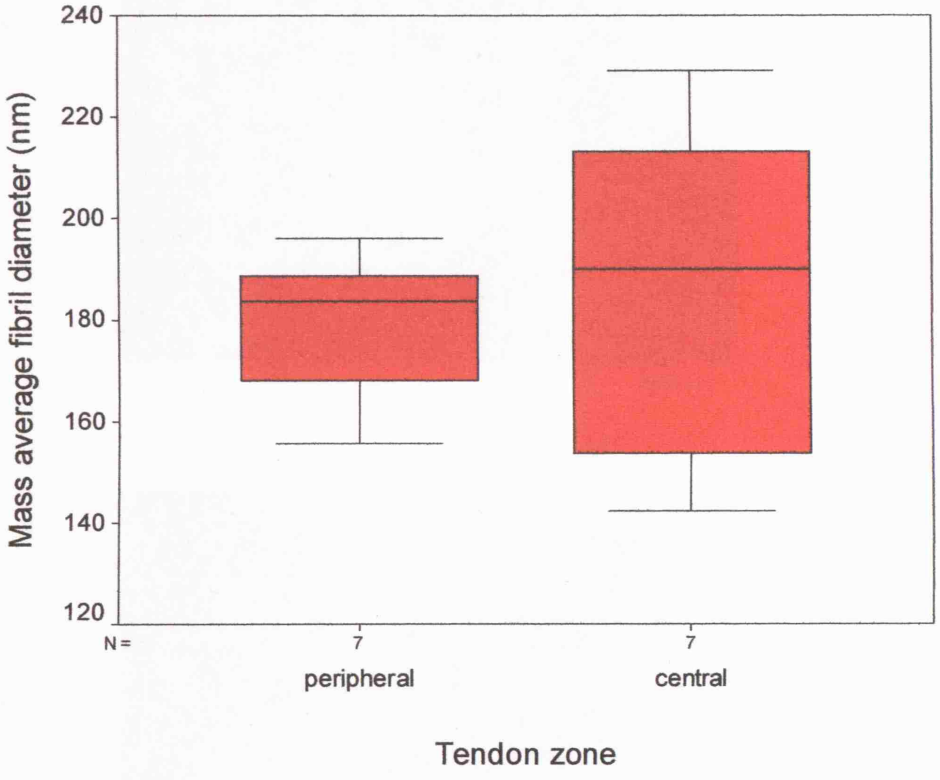
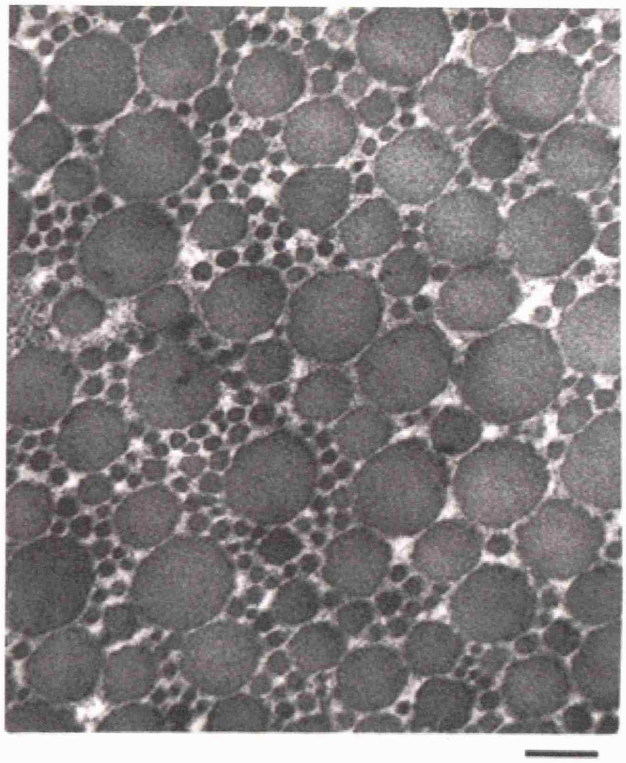
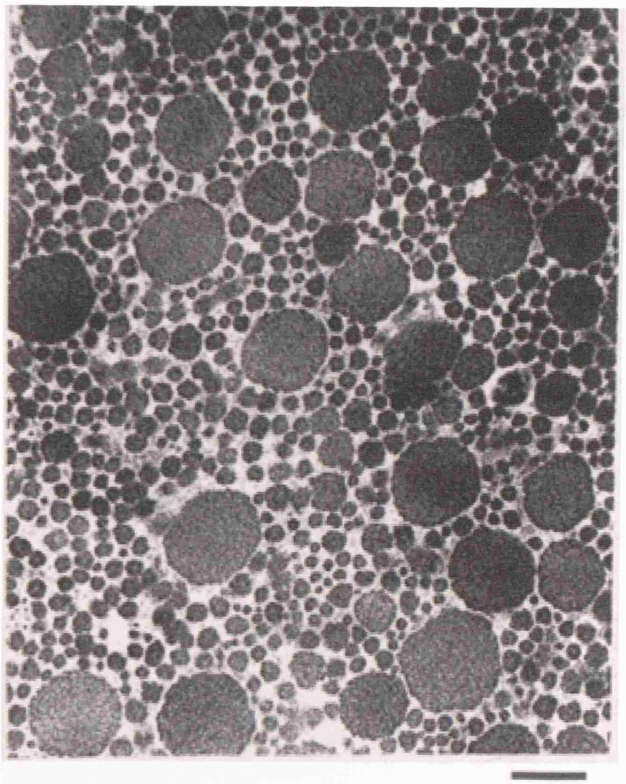


Figure 6.11: Boxplot showing mass average collagen fibril diameter (MAD) in central and peripheral zones of the SDFT. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median).



Young SDFT



Old SDFT

Figure 6.12: Electron micrographs of central region collagen fibrils from young (7 years) and old (20 years) horse tendon. Note the increased proportion of small diameter collagen fibrils in the old SDFT when compared to the young SDFT (Bar represents 200 nm).

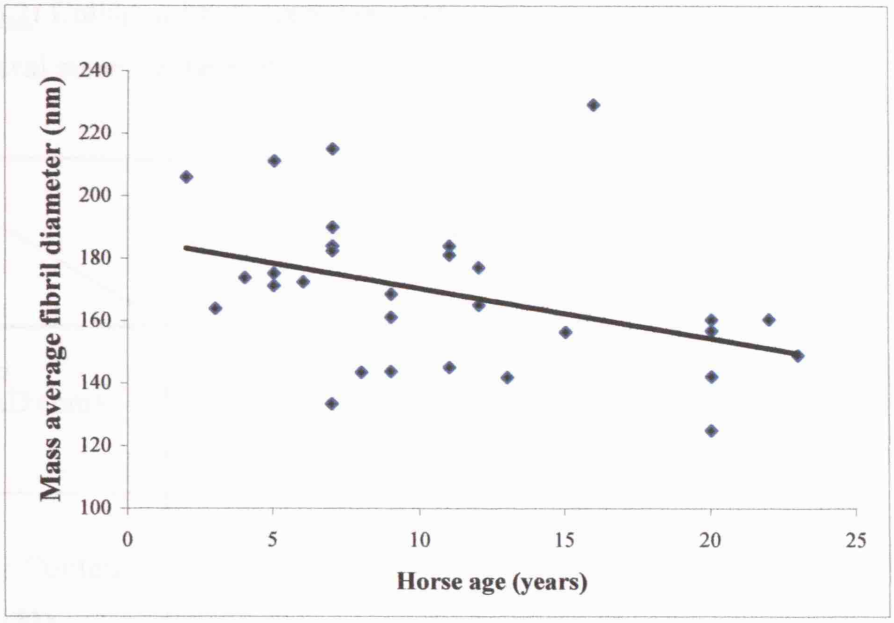


Figure 6.13: Mass average fibril diameter (MAD) values in the central region of the SDFT against horse age (R^2=0.153, n=30, p=0.033).

Table 6.2: Collagen fibril diameters and matrix composition of central and peripheral zones of the SDFT.

	SDFT Central Zone	SDFT Peripheral Zone
MAD (nm)	185 ± 35.5	178.4 ± 15.9
Water Content (%)	65.3 ± 2.5	65.4 ± 1.7
Total Sulphated GAG (μg/mg)	8.04 ± 2.3	7.73 ± 2.2
Collagen Content (%)	86.1 ± 4.8	84.4 ± 5.2

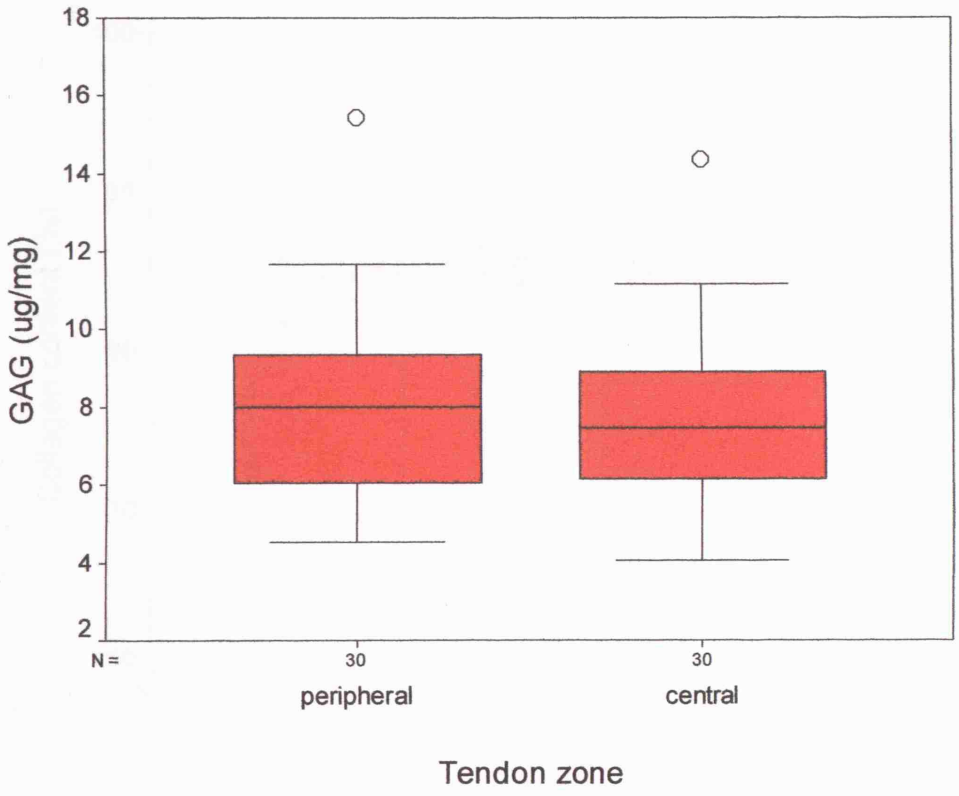


Figure 6.14: Boxplot showing glycosaminoglycan (GAG) concentration in central and peripheral zones of the SDFT. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. ^{o} represents an outlier value).

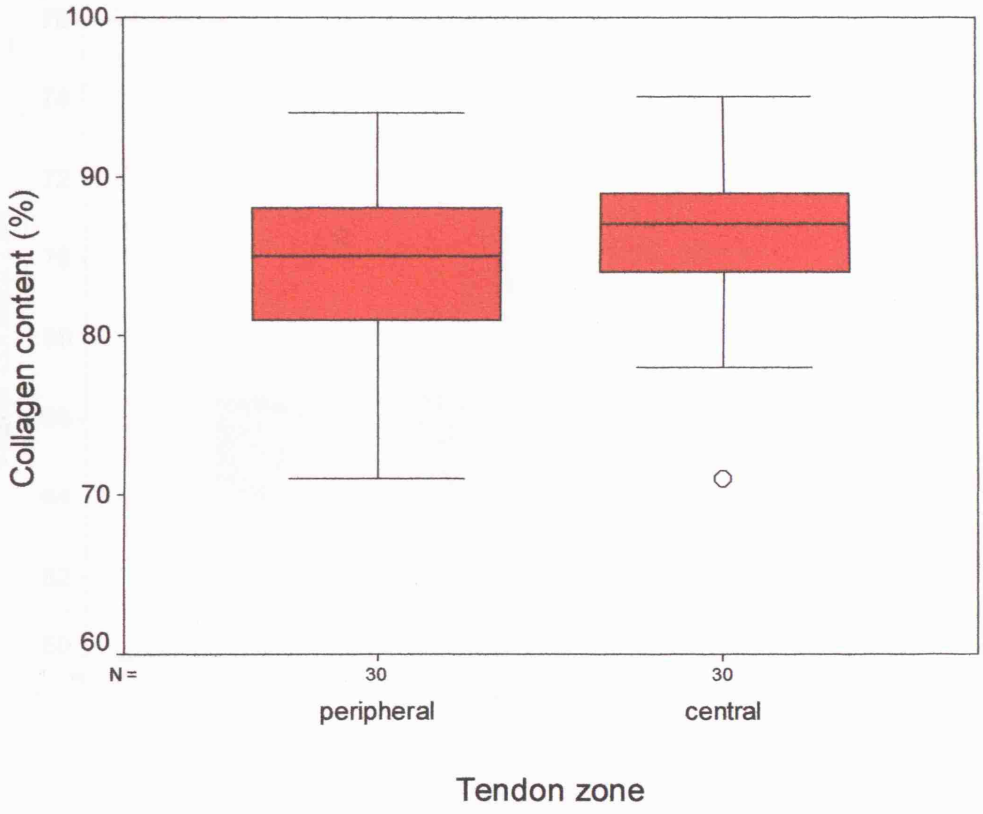


Figure 6.15: Boxplot showing collagen content (%) in central and peripheral zones of the SDFT. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. ° represents an outlier value).

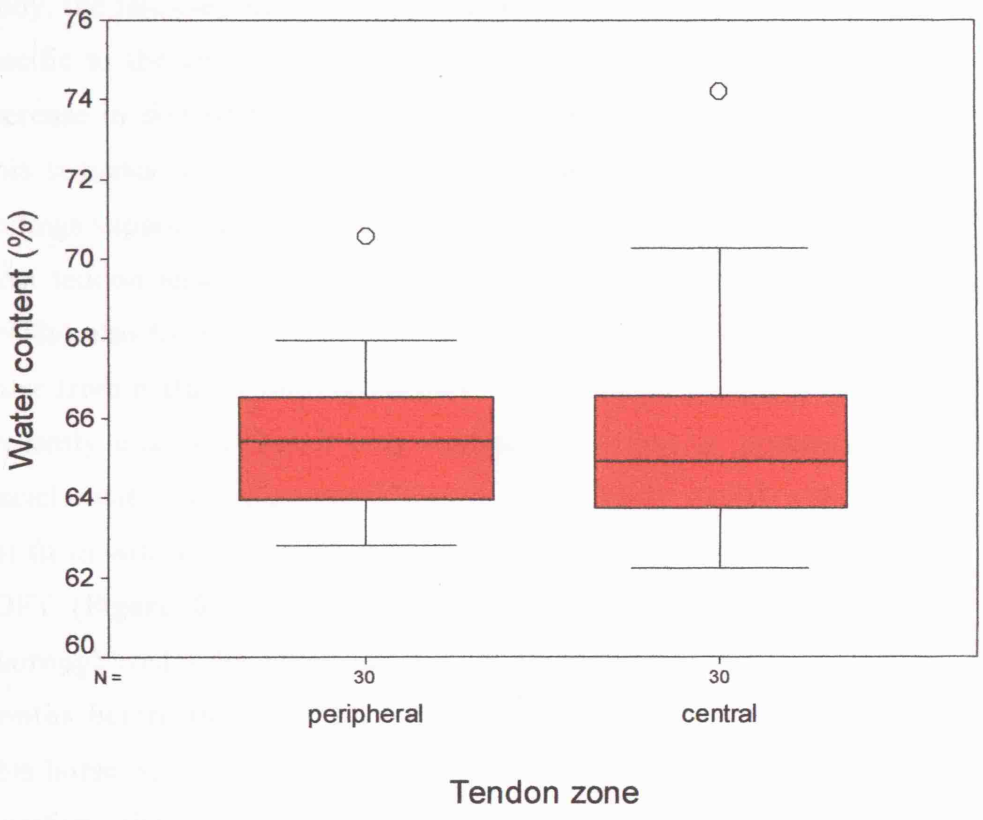


Figure 6.16: Boxplot showing water content (%) in central and peripheral zones of the SDFT. (The box represents the interquartile range containing 50 % of the values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. ° represents an outlier value).

6.6 DISCUSSION

6.6.1 Age Related Changes to Tendon Morphology

The results of this study demonstrate age related changes to both tendon fascicle and fibril morphology. It was found that the greatest age related change was observed in the central core of the SDFT. In the tendons examined in this study, the fascicles underwent a decrease in size with increasing age, which was specific to the central core of the tendon. Collagen fibrils also underwent a decrease in size with increasing age specific to the central core of the SDFT. This suggests that these changes may weaken the core of the tendon. These findings support the work of Marr *et al.* (1993) as it has been documented that most tendon lesions develop in the central region of the SDFT. Parry *et al.* (1978a) also found that collagen fibril diameters decreased in the SDFT of the horse from maturity until senescence. The cause of the decreased fascicle size is presently unknown but it may represent splitting or degeneration of tendon fascicles with increasing age (Dowling *et al.*, 2000). In our study, one horse did not fit in with the trend of decreasing collagen fibril diameters with age in the SDFT **(Figure 6.13)**. This horse was a 16 year old mare, 16.3 hh, Irish Thoroughbred - Ex show jumper (B Grade) who had show jumped until 3 months before the horse had been destroyed for reasons relating to old age. This horse was the only show jumper in the study and a heavier type of horse, therefore, the horse had undergone a different type of exercise to the other horses, which may have contributed to it being an outlier.

In the present study, the spacing of the fascicles and overall size of the tendon was not significantly different. These data suggest that ageing results in an increase in the number of fascicles in the central core, which may be due to fragmentation, or remodelling and may pre-dispose this part of the tendon to mechanical overload. Gillis *et al.* (1997) found that central region fascicles were significantly larger than fascicles in other regions and that fascicle CSA decreased significantly with increasing horse age. The results of our study support these findings although our fascicle CSA measurements were slightly lower but this is most probably because different sampling regions were used

good quality transverse sections. Our results are in agreement with previous research by Dowling *et al.* (2000) who has shown that the CSA of fascicles in the SDFT are in the range of 0.8-1.2 mm^2 and that they can be seen at low magnifications using SEM. Our results demonstrated that fascicles in the central region (0.94 ± 0.06 mm^2) of the SDFT were larger in all horses aged 2-23 years and that fascicles in the medial peripheral (0.42 ± 0.08 mm^2) and lateral peripheral (0.38 ± 0.09 mm^2) regions were smaller in size **(Figure 6.9)**. Thus, fascicles in the central region appear to start off being larger than those in the medial peripheral and lateral peripheral regions and continue to be larger throughout maturity until senescence.

The irregular shape of fascicles can make identification and measurement of fascicles difficult. In addition to measuring fascicles in the SDFT, it was originally proposed to measure the fascicle CSA of the DDFT, SL and the CDET. Unfortunately this was not successful due to the fact that the DDFT and SL tendons were too large to view complete fascicles under the SEM used, even at the lowest magnification. The SL also contained muscle, which made distinguishing fascicles difficult. It was also not possible to measure fascicle CSA in the CDET as identification of fascicle boundaries made measurement difficult. Further work is therefore needed to improve on our SEM technique.

In relation to the fact that the CSA of the whole tendon did not change with age in this study, these findings support the work of Haut *et al.* (1992) who found no correlation between age and tendon CSA in the patellar tendon from dogs 0.5 to 15 years old. Gillis *et al.* (1995) also found no correlation between age and tendon CSA in a study on the SDFT of horses ranging in age from 2 to 23 years and it was thought that any changes in CSA evident represented adaptation to increased load bearing rather than maturation.

Previous studies have found age related changes to the crimp pattern in the core of the tendon with ageing (Patterson-Kane *et al.*, 1997b), which would pre-dispose this part of the tendon to mechanical overload and these may be associated with the changes in fascicle and fibril organisation found in this

horses show changes to SDFT fascicle and fibril morphology that are likely to predispose them to site specific tendon degeneration, which may be related to reduced collagen turnover in the central region of the tendon.

The results of this study also demonstrate a difference in matrix organisation between central and peripheral zones of the SDFT. Tendon fascicle and fibril morphology varied in relation to both the site within the SDFT and the age of the horse. Therefore, fascicles were larger in the central core of the tendon at all ages but this was not due to a difference in diameter of the constituent collagen fibrils. Previous studies have also found no difference in the collagen fibril diameter between central and peripheral regions of the SDFT of young and old horses (Birch *et al.*, 1999b). In a study by Magnusson *et al.* (2002) investigating collagen fibril diameters in ruptured and intact Achilles tendons it was shown that the collagen fibril distribution showed no region-specific differences in either the ruptured or intact tendons. However, there did appear to be a site-specific loss of larger fibrils in the core and periphery of the Achilles tendon rupture site.

In relation to the septal width measurements obtained in this study, there was little variation in septal width found from region to region or from young to old horses. These results support the work of Gillis *et al.* (1997) who also found no regional or age effects on septal width in the SDFT of horses ranging in age from 2 to 23 years. Previous research by Josza *et al.* (1991) has also shown through SEM and TEM studies that there is little variation in density of endotenon in tendons from humans aged 11 to 53 years.

6.6.2 Effects of Age and Exercise on Collagen Fibril Diameters and Material Properties

Changes in collagen fibril diameters may be caused by tendon functionally adapting to its biomechanical environment and factors most probably influencing this are horse age and the effects of exercise. Cherdchutham *et al.* (2001) demonstrated that collagen fibril diameter distribution, which influences

amount and type of exercise provided to immature horses. Flexor tendons in horses are often submaximally loaded under physiologic conditions, so optimal tendon strength is a prerequisite to prevent injury. In the present study, there was a significant correlation between elastic modulus, ultimate stress, MAD and horse age. Both the elastic modulus, ultimate stress and MAD decreased significantly with horse age. Age, horse breed, and exercise level are known to have an influence on the mechanical properties of tendon (Birch *et al.*, 1999a; Crevier *et al.*, 1996; Crevier-Denoix *et al.*, 1998; Gillis *et al.*, 1995; Kasashima *et al.*, 2002b). The group of horses used in this study ranged in age from 2-23 years, and they were a mixture of Thoroughbred, Thoroughbred-type and non-Thoroughbred adult horses all of which had varying exercise levels **(Table 3.1, Chapter 3)**. Interestingly, when the horses were grouped into these three horse types, there were only differences seen in the Thoroughbred group in relation to the fact that elastic modulus decreased significantly and ultimate stress decreased significantly with increasing horse age. There were no correlations found between ultimate stress and elastic modulus with horse age in either the Thoroughbred-type or non-thoroughbred groups. It therefore appears that the Throughbred group of 14 horses may be causing the age related decrease in material properties of the SDFT found in this study. This is most probably due to Thoroughbred horses being specifically bred for racing and having the genetic make-up to enable them to perform as equine athletes compared to other horse breeds. The group of Thoroughbred horses in the present study would have had the highest level of exercise compared to the other groups, therefore, the age related decrease in material properties appears to have been accelerated by exercise. However, the effect of genotype on Thoroughbred horses has not yet been investigated. Genetic potential can be increased by selective breeding, however it has been suggested that in the case of the racing Thoroughbred, due to gene pool limitations, the maximum improvement in genetic performance has already been attained (Hill, 1988). In the present study, it appears that other factors may be influencing mechanical properties of the SDFT, such as different training regimes and levels of exercise in Thoroughbred horses.

significant effect on the material properties of tendon. Some studies have reported an increase in mechanical properties (Haut *et al.*, 1992; Thermann *et al.*, 1995), some have reported no change (Batson *et al.*, 2003; Lewis & Shaw, 1997), while others have reported a decrease in mechanical properties with age (Birch *et al.*, 2001). In our study, elastic modulus decreased as horse age increased, due to the fact that tendons appear to have become less stiff (i.e. loss in elasticity) with age. In contrast, in a study by Gillis *et al.* (1995) investigating the effect of maturation and ageing on material and ultrasonographic properties of the SDFT, a significant positive correlation was found between elastic modulus and age. However, in this study the increase in elastic modulus can be explained due to the fact that the group of 2 year old horses were compared to the group of horses 3 years and older. Therefore, the effects of maturation were taken into account but the elastic modulus was not evaluated over an age range. In the present study, horses from an age range of 2-23 years were used and it was shown that the elastic modulus decreased with increasing horse age due to the fact that maturation through to senescence stages of development were investigated. Other authors have also shown in other species that that flexor tendon strength, energy conservation and elastic modulus increase significantly during maturation and decrease from maturation to senescence (Danielsen & Andreassen, 1988; Elliot, 1965; Nathan *et al.* 1978; Vogel, 1983; Walker, 1976).

6.7 CONCLUSIONS

- As a horse ages it shows an increase in the proportion of small diameter collagen fibrils and a reduction in elastic modulus (material stiffness) in the SDFT.

- Fascicle size and collagen fibril diameters decrease in the central core of the SDFT with increasing age, the site where degeneration and subsequent injury most commonly occurs.

- Fascicle size shows a significant difference between regions of the SDFT whereas collagen fibril diameters show no significant difference between central and peripheral regions.

CHAPTER 7

GENERAL DISCUSSION

7.1 GENERAL DISCUSSION

7.1.1 The relationship between tendon morphology and function

The aim of the present study was to correlate the molecular and morphological characteristics of tendons with their mechanical properties. Results have supported our hypothesis that the mechanical properties of the SDFT can be predicted by the matrix composition and morphology of the tissue. This was shown to be true not only for the SDFT but also for other tendons that have different functions such as the DDFT, SL and CDET. The results of the present study have shown the relationships that exist between functional morphology, matrix composition and mechanical properties of tendon **(Table 7.1, Figure 7.1)**. SDFTs that have a lower elastic modulus and are therefore more easily stretched, have a similar collagen content to SDFTs made of a stiffer material but have smaller diameter collagen fibrils. SDFTs with smaller diameter collagen fibrils have a higher total sulphated GAG content, which may be responsible for preventing further lateral growth of the fibrils. Tendons with smaller diameter collagen fibrils also have higher water content and it may be possible to detect this using imaging techniques, which would allow a prediction of tendon strength and stiffness to be made *in vivo*. Matrix properties can be determined *in vivo* to some extent using ultrasonography, MRI and biochemical markers. These techniques are becoming more and more sophisticated and informative with time. In the present study, a link has been established between matrix characteristics with mechanical properties of the tendon, which is important to be able to determine tendon vitality. In terms of horse welfare it would be advantageous to reduce the incidence of tendon rupture by being able to predict which horses were likely to suffer a tendon rupture so that the appropriate steps could be taken to avoid injury. Now that the relationships between basic matrix properties and mechanical properties have been ascertained, it is important to determine the factors that regulate these properties so that these can be manipulated to achieve optimal tendon function during locomotion.

Table 7.1: Summary table of results and correlations found in the present study (N/S = non-significant).

	Age	Elastic Modulus	Ultimate Stress	CSA	MAD (central region)	GAG content
Elastic Modulus	Significant decrease with increasing age	X	Significant positive correlation	Significant negative correlation	Significant positive correlation	N/S
Ultimate Stress	Significant decrease with increasing age	Significant positive correlation	X	Significant negative correlation	Significant positive correlation	N/S
CSA	N/S	Significant negative correlation	Significant negative correlation	X	Significant negative correlation	Significant positive correlation
MAD (central region)	Significant decrease with increasing age	Significant positive correlation	Significant positive correlation	Significant negative correlation	X	N/S
Fascicle CSA (central region)	Significant decrease with increasing age	N/S	N/S	N/S	N/S	N/S
Water content	N/S	Significant negative correlation	Significant negative correlation	Significant positive correlation	N/S	Significant positive correlation
Collagen content	N/S	N/S	N/S	N/S	N/S	N/S
GAG content	N/S	N/S	N/S	Significant positive correlation	N/S	X
DNA content	N/S	N/S	N/S	N/S	Significant negative correlation	N/S

The specific function of the SDFT, DDFT, SL and CDET is reflected in the mechanical properties and collagen fibril morphology of each. Collagenous structures within the equine distal forelimb have different structural and material properties. They also have different morphological properties in terms of collagen fibril diameters. An important finding from our work in Chapter 5 is that a significant positive correlation exists between collagen fibril diameters and elastic modulus in energy storing structures (SDFT, SL) and non-energy storing structures (DDFT, CDET) from the equine distal forelimb. Tendons composed of smaller diameter collagen fibrils have a lower elastic modulus (i.e. have a less stiff matrix) **(Table 7.2)**, which may be due to larger diameter collagen fibrils providing a tendon with greater tensile strength while the smaller fibrils are more important for providing the tendon with elasticity. Matrix composition in terms of water content, total sulphated GAG content, and collagen content were also significantly different in the SDFT, DDFT, SL, and CDET. Tendons which are composed of a less stiff material, and are therefore more easily stretched, have a higher water content and higher GAG content than tendons made of a stiffer material but have smaller diameter collagen fibrils.

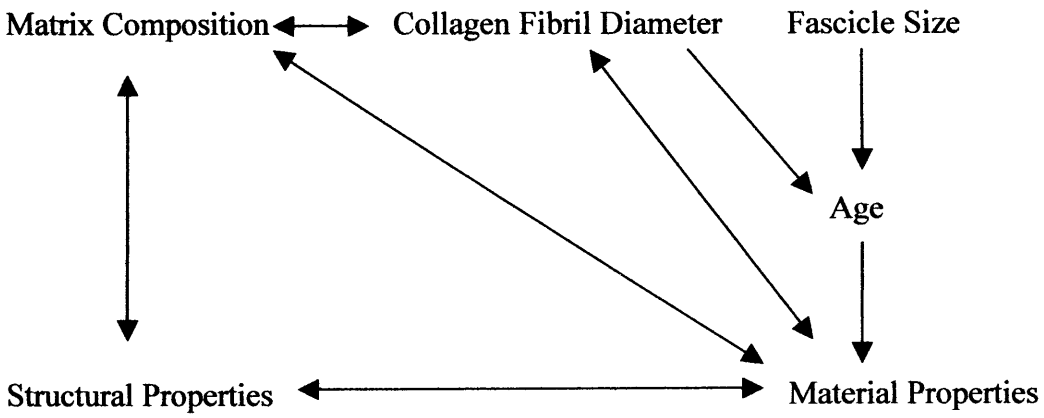


Figure 7.1: Relationships between mechanical properties, morphology and matrix composition of tendon.

Table 7.2: Summary table of material properties and morphology in the different structures of the equine distal forelimb.

Structure	Type of Tendon	Collagen Fibril Diameters	Stiffness
SDFT	Energy storing	Mix of large and small diameter fibrils	Medium
DDFT	Non-energy storing	Mix of large and small diameter fibrils	Medium
SL	Energy storing	Mostly small diameter fibrils	Low
CDET	Non-energy storing	Mostly large diameter fibrils	High

A tendon needs to have the appropriate mechanical properties in terms of stiffness and strength for it to function efficiently. The SDFT and SL need to be highly elastic (low stiffness) in order to stretch and store maximum amounts of elastic energy. The high incidence of injury to the SDFT suggests that tendon strength is not sufficient for mechanical demand. The SDFT acts as an energy store and a pre-requisite for this is that the tendon should stretch elastically (Shadwick, 1990). However, an increase in the CSA of the SDFT would increase tendon stiffness further and reduce strain therefore reducing the energy storing capacity. Tendons that store elastic strain energy are therefore, working within narrow safety margins (Alexander, 1981). The DDFT is subjected to lower stresses and strains (Platt *et al.*, 1994), and is therefore much less important for energy storage. The DDFT is rarely injured and may not have the necessity to increase in strength. The CDET functions as a relatively inextensible link between the extensor muscles and the distal phalanx and an increase in stiffness would not impair this function (Birch *et al.*, 1999a). The CDET requires a higher stiffness in order to transfer muscle force quickly and efficiently, and to reduce the muscular work required to maintain tension in the tendon.

As a result of the present study, an important question arises which is 'Why are tendons that need to be stiffer not just made bigger?' In a study by Batson *et al.*

(2003), the material properties and matrix composition of the SDFT and CDET were investigated. It was shown that the SDFT had a significantly higher cross-sectional area, structural stiffness, failure load, failure strain and lower elastic modulus than the CDET. It appears that the SDFT has increased its strength entirely by increasing its cross-sectional area resulting in a higher structural stiffness relative to the smaller CDET. In our study (Chapter 5) it appears that in addition to the SDFT, the DDFT and SL have also increased their strength entirely by increasing their cross-sectional area resulting in a higher structural stiffness.

A large resilient structure such as the SDFT may store a similar amount of energy to that of a smaller stiffer structure such as the CDET; the SDFT however, is likely to store and release energy more appropriately by deforming over a larger distance when the metacarpophalangeal joint sinks to the ground recoiling during the swing phase. Significantly higher failure strains in the SDFT than the CDET indicate that it can stretch and deform to a greater extent before it fails at maximum load. The SDFT has conflicting requirements for strength and elasticity. Strength is critical to cope with the peak force the tendon receives through locomotive and gravitational forces. However, increasing strength by increasing cross-sectional area will make a tendon stiffer. A thicker, stiffer tendon may store the same amount of energy but this would not easily be recoverable and, therefore, of less use in an energy saving mechanism. It also appears that differences in the matrix composition including water and GAG contents allow the SDFT to remain more elastic as a material (Batson *et al.*, 2003). In addition to this, it appears from our study (Chapters 4 & 5) that differences in collagen fibril morphology also allow the SDFT to remain more elastic as a material due to the presence of small diameter collagen fibrils.

There appear to be two phases in the life of an energy-storing tendon i.e. a growth phase and an ageing phase. At birth, the tendon appears to be 'blank' i.e. homogenous throughout its length and unadapted to load-bearing, in a similar fashion to that suggested for cartilage (Little & Ghosh, 1997; Brama *et al.*, 2000). During the growth phase the tendon develops a tendon matrix which is then influenced by its biomechanical environment. Exercise is necessary to stimulate optimal tendon development, although if the level is excessive during the growth phase it may potentially cause injury to the tendon.

Batson (2002) has shown that the horse forelimb provides an excellent model for investigation of the effect of the postnatal environment on the development of energy-storing and positional tendons. Although the structure and composition of these digit tendons is similar prenatally, within one week postnatally, the energy-storing flexor tendon increased significantly in size. This increased size, led to increased strength and stiffness, in association with a rapid increase in body weight. The extensor tendon however, did not increase in cross-sectional area, but altered the properties of the constituent matrix in order to increase strength. If the cues required for initiating changes to the matrix of the CDET postnatally could be identified and applied to the SDFT to improve/alter the quality of the matrix, there would be huge implications for both tendon healing and tissue engineering. Understanding how and why the CDET is able to adapt may be the key to designing training regimes for young horses to prevent tendonitis in the future. Tissue engineered constructs to replace injured tendons need to initiate the formation of a structure close to the original, and the mechanical cues required for this are tissue specific. Tenocytes from foetal tendon show the potential to follow particular pathways given the specific cues and may have potential future use in tissue engineered constructs (Batson, 2002).

Structural and material properties vary widely between the SDFTs of different horses (Chapter 4). In particular, there is a wide range in elastic modulus between different horses with some being almost twice as stiff as others. It was of particular interest based on these findings to then investigate whether some horses have an inherently weak musculoskeletal system and are therefore more prone to injury. To do this, it was interesting to compare whether those horses in our study that had weak tendons also had weak bones. This was done by mechanically testing the metacarpal bones of these horses, which were collected at post mortem and stored at –20 °C. Results from our group by Draper *et al.* (2004), demonstrated a significant positive correlation exists between bone tissue strength and tendon tissue strength within the same individual. Horses with weak bone tissue also tended to produce weak tendon tissue. Similarly there are individuals with strong bone and tendon tissues. This data suggests that an important component of this correlation in strength will be due to the quality of the type I collagen, which is a major constituent of both. Further studies are currently underway to investigate this.

An interesting question to address is 'Does the SDFT function efficiently during locomotion?' SDFTs which are composed of a less stiff material have smaller diameter collagen fibrils than tendons composed of a stiffer material (Chapter 4). According to Smith *et al.* (2002), this particular tendon contributes to energetic efficiency in an animal evolved and further selectively bred for high-speed locomotion, therefore the SDFT functions close to failure with a low safety margin. The SDFT is subjected to very high loads of 10-20 kN (Goodship *et al.*, 1994) and stretches up to 16 % *in vivo* during high-speed locomotion (Stephens *et al.*, 1989). This is very close to its failure strain *in vitro* which has been reported to be between 12-20 % (Goodship *et al.*, 1994). This may explain the extremely high incidence of partial rupture of this specific tendon. Once tendons are injured, the slow process of repair with fibrous scar tissue occurs. This scar tissue is essentially the same as that forming in skin wounds and does not have the same matrix components as the normal tendon. Useful energy recovery is associated with a more elastic tendon. As the SDFT of the horse has to essentially function as a spring to store energy on loading, tissue stiffness is critical for optimising this function. Too stiff or too elastic a 'spring' would make it less efficient for the given weight of animal. It is therefore possible that, once an optimised tendon is formed at skeletal maturity, there is a much reduced need to synthesise further matrix, and so the synthetic machinery is 'turned off' (Smith *et al.*, 2002).

The control of expression of genes for the matrix protein composition in the tendon during growth, exercise and ageing is not clear. Tendons exhibit extracellular matrix remodelling in response to mechanical stimuli (Benjamin & Ralphs, 1998; Malaviya *et al.*, 2000). Several *in vitro* studies have shown remodelling to be mediated via tenocytes which can detect and respond to various mechanical stimuli through mechanotransduction (the conversion of a mechanical stimulus into a cellular response) pathways (Banes *et al.*, 1995; Ehlers & Vogel, 1998; Evans & Trail, 2001; Ralphs *et al.*, 2001; Zeichen *et al.*, 2000). However, the precise stimuli involved in initiating mechanotransduction pathways have not been fully established. The low level of matrix synthesis in tendon during growth, exercise and ageing could be a consequence of cellular senescence, loss of mechanotransduction, or growth factor stimulus (Smith *et al.*, 2002). Normal differentiated cells have a limited capacity for division (Goldstein, 1990; McCormack & Campisi, 1991) referred to as programmed

cellular senescence. However, senescence is not programmed cell death because senescent cells remain viable although they have lost the capacity to replicate DNA (Buckwalter *et al.,* 1993). Studies have shown that immunohistochemical staining of prototypic anabolic growth factor, TGF-β, is lost from tendon fascicular tissue after skeletal maturity, furthermore, there is no detectable TGF-β gene expression after birth (Cauvin *et al.,* 2000; Cauvin, 2001). These findings are most consistent with a mechanism more related to the absence of cellular synthetic ability, although a reduction in cell activity and the loss of a co-ordinated mechanotransduction response (such as the loss of gap junctions between cells) could also contribute (McNeilly *et al.,* 1996). If the major effect on the synthetic ability of the tendon proves to be the lack of appropriate growth factors, this may provide a possible therapeutic direction for the prevention of tendon degeneration.

Riley *et al.* (2002) have demonstrated there is evidence for increased turnover of the matrix during tendon degeneration, potentially mediated by an alteration in the balance of some members of the matrix metalloproteinase (MMP) family relative to their specific inhibitors, TIMPs (tissue inhibitors of matrix metalloproteinases). Modulating this enzyme activity may therefore have therapeutic potential, although broad-spectrum inhibitors of MMPs are known to cause tendinopathy after prolonged treatment (Riley, 2000). MMP-3 is a MMP with a wide substrate range that includes most extracellular matrix components and it is also a potent activator of other MMPs (van Meurs *et al.,* 1999). Thus, it has the potential to play a major role in regulation of tendon extracellular matrix degradation and tissue remodelling (Ireland *et al.,* 2001). Riley (2000) has demonstrated that tenocytes produce both the components of the extracellular matrix and also the enzymes that degrade them. They also produce cell adhesion molecules that mediate cellular interactions and influence cell signalling, transcription and response to growth factors. Mis-expression of the matrix or adhesion molecules is the underlying cause of many developmental or degenerative disorders (Lukashev & Werb, 1998; Petruzzelli *et al.,* 1999). Understanding the control mechanisms of these cellular activities may lead to therapies to promote the repair/regeneration of the tendon matrix.

7.1.2 Tendon and Ligament Reconstruction – How does collagen organisation alter mechanical function?

Tendon and ligament injuries are relatively common (Butler *et al.,* 2004; Woo *et al.,* 2000b). These injuries include Achilles tendon rupture (Maffulli, 1999), partial tears and inflammations of the rotator cuff complex (Ruotolo & Nottage, 2002), rupture of the patellar tendon (Crossett *et al.,* 2002), and rupture of the anterior cruciate ligament (ACL) (Woo *et al.,* 2000b). The findings from Chapters 4 & 5 of the present study have a clinical relevance in relation to replacements used in tendon reconstruction procedures. Tendon and ligament injuries are of particular concern because they heal slowly and form scar tissue with poor mechanical properties, therefore there is often a need for reconstructive surgery. Repairs can be conducted using allograft (graft from different site/cadaver) (Crossett *et al.,* 2002), autograft (graft from same human/animal/specimen) (Spindler *et al.,* 2004), and there is also recent interest in using a bioengineered construct (Butler *et al.,* 2000; Guilak *et al.,* 2003). The aim of reconstructive surgery is to restore the mechanical properties and therefore function of the tendon or ligament. For example, the anterior cruciate ligament is often repaired surgically with an autograft consisting of a graft from the patellar tendon or hamstrings. Nonsurgical management of a ruptured ACL is successful for some patients, but in most patients has led to poor results (Hirshmam *et al.*, 1990). The current trend is toward surgical reconstruction with a replacement graft. Unfortunately, there has been a 20-25 % less than satisfactory result associated with ACL reconstruction (Aglietti *et al.,* 1997; Kartus, *et al.,* 1999; Ritchie & Parker, 1996). Thus, it is important to thoroughly understand the function and biomechanics of the role of the ACL in providing stability to the knee joint. According to Woo *et al.* (2000b), many tissue grafts, in autograft and allograft forms, have been used for ACL reconstruction. Currently, the most popular grafts are bone-patellar tendon-bone (BPTB), and quadruple-strand semitendinosus and gracilis tendons (QSTG). The central third of the patellar tendon (8-10 mm), which is harvested with bone blocks on either end for solid fixation, is chosen due to its relatively high stiffness and strength as well as the possibility for bone-to-bone fixation in the tunnels (Arnoczky *et al.,* 1986; Butler *et al.,* 1986).

Achilles tendon rupture generally occurs in the occasional athlete who ruptures the tendon during an explosive push-off manoeuvre (Kannus, 1997). Most frequently, these athletes are middle-aged males who are involved in only intermittent athletic activities (Clement *et al.,* 1984). However, this injury has also been seen in young, high-performance athletes (Woo *et al.,* 2000b). Possible etiologic factors that lead to Achilles tendon rupture include a sudden overload strain, a direct blow to the tendon during contraction, poor blood supply 4.5 cm proximal to insertion, chronic degenerative changes, shear stress between the gastrocnemius and soleus tendons, and steroid injections (Maffulli, 1999; Singer & Jones, 1986). According to Woo *et al.* (2000b), currently the main objective of treatment is to restore the tendon to normal length and strength. However, controversy exists as to whether the ruptured tendon should be surgically repaired or treated nonoperatively. In recent years, athletes and young people who are seeking delayed treatment for an Achilles tendon rupture are treated operatively, whereas acute ruptures in nonathletes are treated nonoperatively (Maffulli, 1999). Maffulli (1999) has shown that when the ruptured tendon is examined surgically, two very consistent findings are noted. The tendon ends are dishevelled and disrupted for several centimetres on either side of the rupture, making surgical repair difficult. In addition, plantar flexion of the foot brings the ends of the tendon into contiguity. Both of these findings point toward nonoperative treatment (Singer & Jones, 1986). However, a very high incidence of re-rupture has been found in the nonsurgical group when compared to patients who were treated surgically (Inglis *et al.,* 1976). According to Maffulli (1999), in addition, those who did not experience re-ruptures seemed less satisfied than the patients treated surgically. Therefore, operative treatment may produce better functional results but may have a higher risk of postoperative complications, whereas nonoperative treatment may result in a less optimal functional result. Finally, another method of treatment for Achilles tendon rupture is a tendon transfer. This has been found to be advantageous in the treatment of old, unhealed ruptures or cases of re-rupture following prior nonoperative or operative treatment (Turco & Spinella, 1986).

Partial tears and inflammations of the rotator cuff complex are relatively common (Kannus & Natri, 1997). These injuries often occur during sudden shoulder movements involved in sports such as tennis, baseball, volleyball, and weightlifting

(Budoff *et al.*, 2003). According to Fukuda (2003), injuries are more common in young active people and nonoperative treatments such as physiotherapy and steroid treatment are most conventionally used for partial tears and inflammations. In more severe cases operative treatment will be performed involving suturing the tear combined with MRI/CT scanning, although complete tears of the rotator cuff complex are very rare.

The present study has shown in Chapter 5 that different tendons have specific requirements in terms of their mechanical stiffness to ensure physiological function. Although the ultimate strength of a replacement is important to prevent re-rupture, the correct mechanical properties within the physiological range of loading are also essential to restore normal function. A reconstructed structure may adapt to its new environment because it will be repopulated by cells, which may lead them to proliferate and remodel that structure. Fibroblasts could migrate into the tendon and then become tenocytes. However, a tendon or ligament without the correct mechanical properties may not have the correct mechanical stimulus provided, so it would not be expected to remodel to the appropriate properties in its correct environment.

An important finding from the present study is that the collagen component and the way in which it is organised is responsible for the mechanical properties of a tendon, and therefore differences in the arrangement of collagen are evident in tendons with different functions. Chapter 5 has shown that tendons with different functions have a specific material stiffness, which correlates with the collagen fibril diameters. Findings from this study show that tendons which are composed of a less stiff material have smaller diameter collagen fibrils than tendons composed of a stiffer material. Those tendons with larger collagen fibril diameters also have a lower water and GAG content. This difference in material properties and matrix composition relates to physiological function, therefore, when carrying out reconstructive surgery these properties of the replacement construct or graft should be considered to ensure restoration of normal physiological function. Future work to determine the mechanisms that control collagen fibril diameters and water content will aid in the design of bioengineered constructs. It would also be interesting in the future to determine the mechanical and matrix properties of the ACL and patellar tendon, as

the ACL is currently repaired surgically using an autograft consisting of a graft from the patellar tendon or hamstrings, therefore it would be important to determine these properties of the replacement construct.

7.1.3 Tissue Engineering – Are stem cells the way forward?

Promising new bioengineering technologies such as tissue engineering, may provide novel tools for reconstructive surgery. Tissue engineering allows the isolation of living, healthy cells from the body to expand under cell culture conditions, and to then combine them with biocompatible carrier materials and retransplant them into patients (Ringe *et al.,* 2002). Tissue engineered transplants are mainly composed of two components: (1) tissue-specific cells, and (2) a biocompatible carrier scaffold on which these cells can develop (Caplan & Bruder, 2001). The cells are important for the production of new tissue through extracellular matrix synthesis and are responsible for the long-term stability of this matrix (Bonassar & Vacanti, 1998). The scaffold material provides short-term mechanical stability of the transplant and provides a template for spatial growth of the developing tissue (Schultz *et al.,* 2000; Sittinger *et al.,* 1996). Therefore, the interaction of scaffolds and cells, cell adhesion on the matrix surface, cell maturation, extracellular matrix production, and cell proliferation are all important for the success of engineered transplants (Ringe *et al.,* 2002).

The prerequisite for tissue engineering applications is the successful isolation and selection of organ specific cells from small tissue biopsies **(Figure 7.2)** (Bonassar & Vacanti, 1998). The *ex vivo* cell expansion in standard culture flasks or bioreactors requires appropriate physiological culture conditions such as temperature, partial oxygen and carbon dioxide pressure, pH, medium supply, and metabolite elimination (Sittinger *et al.,* 1997).

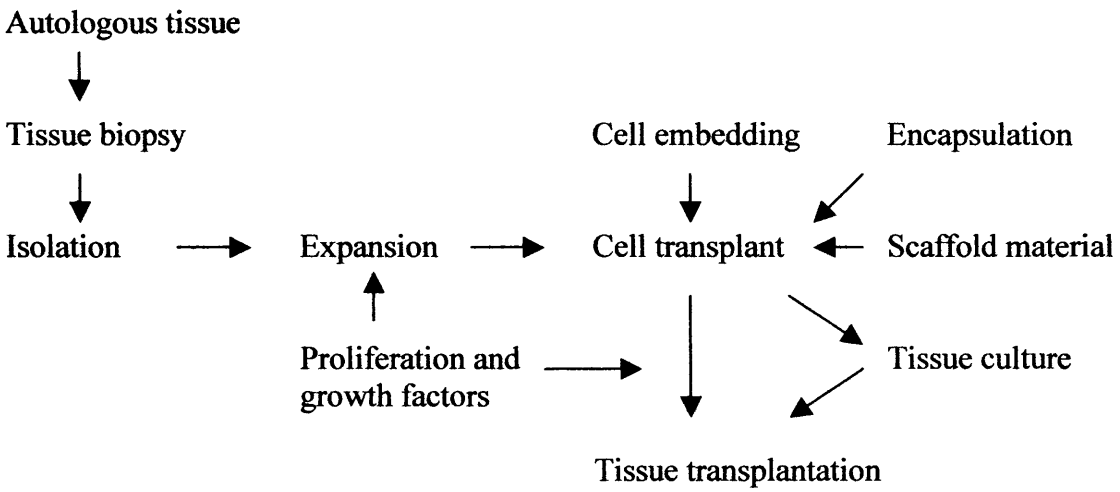


Figure 7.2: General procedure for autologous tissue replacement. Prerequisite for tissue engineering applications is the isolation and selection of autologous cells from tissue biopsies. Following *ex vivo* expansion requires appropriate physiological culture conditions. Expanded cells are embedded into suitable biomatrices. Cell/material composites are either cultured for a while in perfusion chambers or are directly implanted into tissue defects (Adapted from Ringe *et al.*, 2002).

The adult bone marrow stroma contains a subset of non-haematopoietic cells referred to as mesenchymal stem cells (MSC). In response to specific factors and signals, adult stem cells can differentiate and give rise to functional tissue specialised cells. MSC have the potential to differentiate and develop either *in vivo* or *in vitro* into various mesenchymal tissues, including bone, cartilage, fat, muscle, tendon and ligament. MSC can also be relatively easily isolated from different tissues such as bone marrow, fat and muscle. Therefore, these cells provide an attractive source for tissue engineering approaches in the field of orthopaedics.

MSC harbour the potential to develop into tenocytes (Young *et al.*, 1998). Several animal studies and some human clinical trials have evaluated the therapeutic utilisation of MSC in tendon repair. Achilles tendon rupture is currently treated by surgical repair followed by rehabilitation, or nonsurgical treatment combined with prolonged immobilisation (Maffulli, 1999). Both techniques are accompanied by a relatively high rate of complications, including tendon rerupture, infection, wound breakdown and necrosis (Maffulli, 1999). Therefore, the autologous tissue

engineering approach may be appropriate for tendon and related ligament repair (Ringe *et al.*, 2002). One of the first approaches was to implant collagen scaffolds into rabbit tendon and ligament defect sites (Kato *et al.*, 1991). These scaffolds provide early mechanical stability and promote cellular infiltration and native tissue ingrowth. Results showed that rapid repair is achieved with a carbodiimide-cross-linked collagenous implant with the formation of neotendon 10 weeks after implantation, which had a structure and mechanical properties that are similar to those of an autogenous tendon graft. In more innovative applications, a study by Huang *et al.* (1993), seeded human dermal fibroblasts onto type I collagen gels to improve tendon and ligament repair. Composites consisting of fibroblasts and collagen contracted over time *in vitro* and the fibroblasts changed their shape and orientation. Rabbit bone-marrow-derived MSC (Young *et al.*, 1998, Awad, *et al.*, 2000) have also been seeded onto type I collagen gels to improve tendon and ligament repair. In a study by Young *et al.* (1998), autologous, culture-expanded rabbit MSC were seeded onto type I collagen gels that were subsequently contracted onto pretensional polyglycolid acid sutures. After 40 hours of incubation *in vitro*, the composites contracted to 30 % of their original diameter. The cells within the constructs reoriented and exhibited an elongated morphology. Composites augmented with an increased amount of MSC showed more well aligned and elongated cell nuclei. These changes in the nuclear morphology of the cells, caused by physical constraints provided by the contracted collagen fibrils, may induce the fibroblastic lineage and trigger the biosynthetic activity (Awad *et al.*, 2000). MSC collagen composites were then implanted into the long gap defects in the rabbit Achilles tendon (Young *et al.*, 1998). Biochemical and histological analysis revealed improved biomechanical properties, tissue architecture, and functionality of the tendon after injury. These changes were correlated with cell proliferation (Macieira-Coelho & Azzarone, 1990), protein synthesis (Ben-Ze'ev *et al.*, 1980) and extracellular matrix formation (Klebe *et al.*, 1991). However, complete healing of tendon and ligament defects needs additional research that might enhance the repair quality. Likewise in bone and cartilage tissue engineering, the use of exogenous growth factors may improve tendon and ligament tissue engineering. MSC may migrate between the ruptured ends of the defect and then differentiate to form regenerated tendon tissue. The regeneration process is directed by mechanical stimuli and is controlled by growth factors, for example, GDF-5, GDF-6 and GDF-7

(Aspenberg & Forslund, 1999). These growth and differentiation factors (GDFs) have been shown to induce a ligament- or tendon-like tissue after intramuscular implantation in rats (Ringe, *et al.*, 2002).

Autologous equine MSC have also been successfully isolated and implanted from bone marrow into a damaged SDFT of the same horse as a potential novel treatment for tendinopathy (Smith *et al.*, 2003). These cells have the potential to produce actual tendon matrix rather than poorly functional scar tissue, as occurs with conventionally managed superficial digital flexor tendinopathy. The signals required to drive MSC towards differentiation into specific cell types (in this case, tenocytes) are still unclear. The poor success with conventional therapies including physical, pharmacological, and surgical treatments (Bertone, 1996; Bramlage, 1992; Henninger, 1992) supports investigation into the use, in equine tendinopathy, of such stem cell therapies, which are generating considerable interest in the human field. However, this technique requires further assessment to determine if such MSC produce tendon, rather than other tissues, especially scar tissue, *in vivo* (Smith *et al.*, 2003).

7.1.4 Do age related changes to tendon morphology account for central core tendon degeneration?

In the present study, a site-specific response to ageing in the SDFT occurs (Chapter 6) which may contribute towards central core degeneration and subsequent rupture of the tendon. Fascicle and fibril size showed an age related decrease in the core of the SDFT, however the spacing of the fascicles and overall size of the tendon was not significantly different. This data suggests that ageing results in an increase in the number of fascicles in the central core, which may be due to fragmentation or remodelling and may pre-dispose this part of the tendon to or be caused by mechanical overload. Fascicular organisation may be more important in determining the 'toe region' of mechanical behaviour because this region involves straightening of the crimp from relaxed collagen fibres, which become straighter as loading progresses. The initial part of the stress-strain curve (toe region) for the central region of the SDFT has been found to change with age and correspondingly it was

found that fascicle size decreased significantly in the central core of the SDFT. These changes, which are specific to the central core of the tendon, will result in the central zone fibres taking a greater load for a given strain and may therefore contribute to central core degeneration. Fascicle size does not relate to the constituent collagen fibril size or stiffness of the structure. A difference in matrix organisation is evident between central and peripheral zones of the SDFT. Fascicles were larger in the central core of the tendon at all ages, but this was not due to a difference in diameter of the constituent collagen fibrils. Our group are currently investigating whether age related changes to the mechanical and matrix properties occur in the human Achilles tendon based on findings in the horse model from the present study. Preliminary data from Birch *et al.* (2001), indicate that ageing results in a decrease in the strength of the tendon tissue in the Achilles tendon (energy storing) and the tibialis anterior tendon (non-energy storing). Determination of the age at which tendon degeneration begins and the effect of exercise on the process are important areas to focus on in the future.

The findings concerning age related changes in the present study are important in the understanding of tendon degeneration. Elastic modulus and ultimate stress were shown to decrease significantly with increasing horse age. Collagen fibril diameters also decreased significantly in size in the central region of the SDFT with increasing horse age (Chapter 6). Due to the significant positive correlation between elastic modulus and collagen fibril diameters in the SDFT it therefore appears that there is an increase in the proportion of small fibrils with age, which may be due to the formation of new collagen. However, collagen-linked fluorescence gives an indication of collagen glycosylation and therefore the age of the collagen present in the tissue. A significant positive correlation has previously been shown between glycosylation levels (collagen linked fluorescence) and horse age in the SDFT (Batson *et al.*, 2003; Birch, *et al.*, 1999b), indicating that no new collagen is formed and there is a low synthesis rate of collagen. An age-related significant decrease in SDFT cellularity (DNA content) has previously been noted (Batson *et al.*, 2003). These findings suggest that the increase in small fibrils is potentially from disagregation of large fibrils rather than the formation of new collagen. The mechanism for this is thought to be either degeneration or through adaptation/remodelling **(Figure 7.3)**. Therefore, results from the present study

(Chapter 6) suggest that if the SDFT has a large proportion of small collagen fibrils, this may be detrimental to tendon function, as the elastic modulus would be correspondingly reduced, since the SDFT requires large diameter collagen fibrils to provide tensile strength to the tendon.

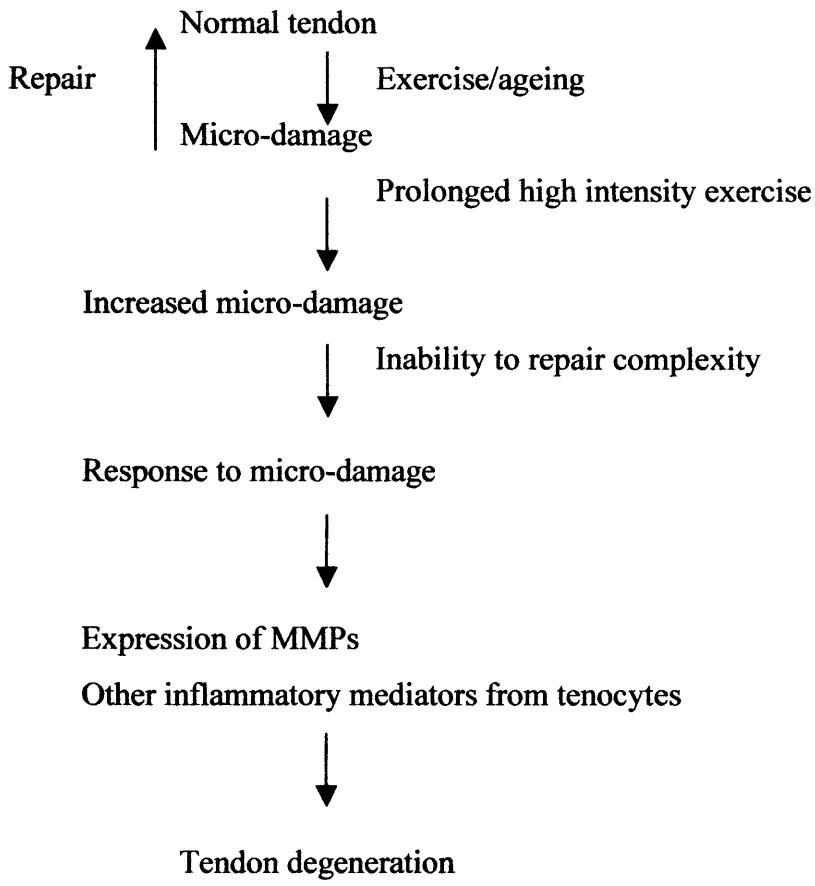


Figure 7.3: Proposed tendon degeneration pathway from the present study.

After skeletal maturity, the energy storing SDFT appears to age, with a decrease in structural and material properties, along with the reduction in collagen fibril diameters and fascicle size in the central region. These changes seem to be accelerated by exercise. Patterson-Kane *et al.* (1997a) showed an increased predominance of small diameter collagen fibrils in the central core region of the

SDFT in the long-term exercised group of horses. These findings suggest that exercise causes disruption of the collagen. Exercise given to the horse serves only to accelerate the ageing effect of microtrauma, resulting in tendon matrix degeneration. The mature tendon normally endures the natural life-style of the horse. It ages and accumulates micro-damage, but only to levels that would not endanger the functional competence of the tissue. However, the artificial superimposition of increased exercise on this ageing mechanism (although training and racing horses, or increased athleticism and longevity in man) potentially accelerates the ageing process to weaken the tissue sufficiently to allow the initiation of clinical tendinopathy when loads overcome the functional capacity of the tissue (Smith *et al.*, 2002).

There are several potential candidates for a mechanism of matrix degeneration. These include direct matrix damage from physical forces, hyperthermia of tendon tissue caused by energy lost as heat during hysteresis (Wilson & Goodship, 1994) and relative hypoxia (Kannus & Jozsa, 1991; Jarvinen *et al.*, 1997; Stromberg & Tufveson, 1969), all of which could act either directly on the matrix or on tenocytes to cause a release of destructive proteolytic enzymes (Smith *et al.*, 2002).

Based on the results from these studies, there was a strategy developed by Smith *et al.* (2002) for the prevention of strain-induced tendinopathies. This involves maximising the quality of tendon prior to skeletal maturity with early introduction of controlled exercise regimens with appropriate monitoring **(Figure 7.4)**. The exact level, quantity and intensity of exercise necessary is currently unknown, but a 'window' of opportunity in both time and amount/intensity probably exists. If a tendon at this stage, responds in a fashion similar to bone, then relatively short periods of high and diverse strain rates would be most appropriate. It also seems important to reduce the degeneration after skeletal maturity by avoiding training directed at tendon adaptation in the adult, as it will serve only to accelerate degeneration, and hence long periods of high peak loads should be avoided. Another possibility is to prevent the cumulative microdamage in adult tendon by identifying and preventing the processes involved (e.g. the release of proteolytic enzymes) and to reactivate resident cell populations to repair microdamage, or at least re-activate matrix synthesis.

The present study has demonstrated that as a horse ages it shows an increase in the proportion of small fibrils, a reduction in stiffness and elastic modulus. Therefore the horse should be more efficient in terms of energy storage, and improve its athletism? However, it appears that alternatively the system is compensating for decreased muscle efficiency or magnitude of loading to maintain the advantages of the elastic storage principle in energy conservation.

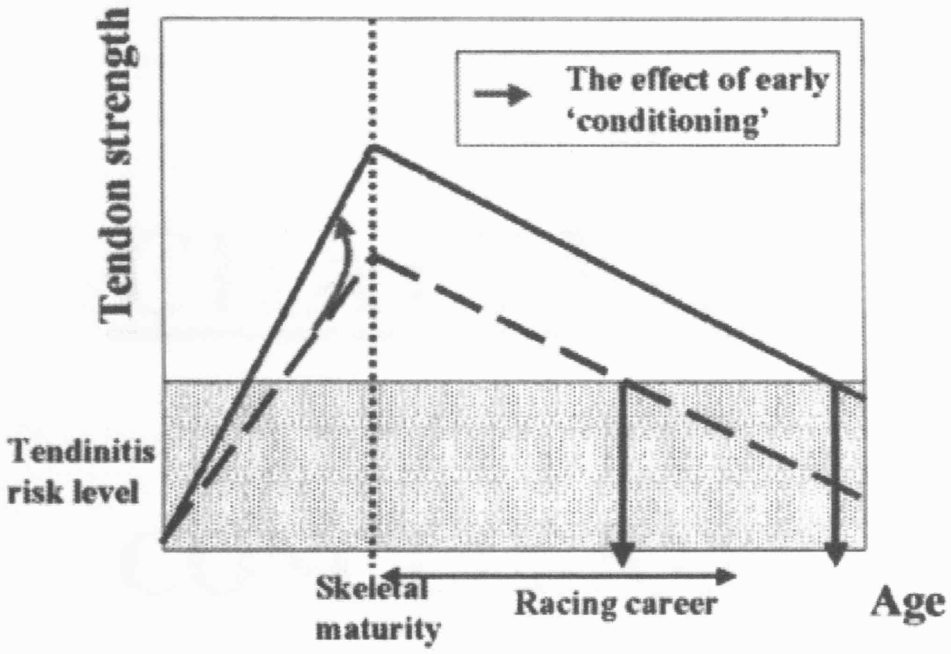


Figure 7.4: Stylised hypothetical response of tendon to the early introduction of exercise. The two dotted lines refer to two separate individuals: one that develops poor-quality tendon during growth (*dashed line)* and the other that develops good-quality tendon (*continuous line).* Both accumulate damage within the tendon associated with post-skeletal exercise and ageing which inevitably and progressively weaken the tendon. However, in the former, this weakening predisposes the individual to clinical tendonitis during its racing career, while the latter is able to survive its racing career without suffering such injury (Smith *et al.,* 2002; with permission).

CHAPTER 8

CONCLUSIONS

8.1 CONCLUSIONS

- Structural and material properties of the SDFT vary widely between horses. SDFTs with a low elastic modulus have smaller diameter collagen fibrils and higher water and sulphated GAG contents.

- The SDFT, DDFT, SL and CDET have significantly different structural and material properties. Morphological properties in terms of collagen fibril diameters and matrix composition are also significantly different between different tendons. A significant positive correlation exists between elastic modulus and MAD for the different structures and within the SDFT. A significant negative correlation exists between water content and elastic modulus, and a significant positive correlation exists between water and GAG content for the different structures and within the SDFT. Tendons which are composed of a less stiff material, and are therefore more easily stretched, have a higher water content and higher GAG content than tendons made of a stiffer material but have smaller diameter collagen fibrils.

- As a horse ages it shows an increase in the proportion of small diameter collagen fibrils and a reduction in elastic modulus (material stiffness) in the SDFT. Fascicle and collagen fibril size showed an age related decrease in the central core of the SDFT, the site where degeneration and subsequent injury most commonly occurs.

CHAPTER 9

FUTURE WORK

9.1 FUTURE WORK

In the present study, a link has been established between matrix characteristics with mechanical properties of the tendon, which is important to be able to determine tendon vitality. In terms of horse welfare it would be advantageous to reduce the incidence of tendon rupture by being able to predict which horses were likely to suffer a tendon rupture so that the appropriate steps could be taken to avoid injury. Now that the relationships between basic matrix properties and mechanical properties in tendon have been ascertained, it is important to determine the factors that regulate these properties so that these can be manipulated to achieve optimal tendon function during locomotion. Our research will now be directed towards minor matrix components that are likely to play a role in modulating collagen fibril and fascicle organisation.

Future work to determine the mechanisms that control collagen fibril diameters and water content will aid in the design of bioengineered constructs. The difference in material properties and matrix composition found in the present study relates to physiological function of a specific tendon, therefore, when carrying out reconstructive surgery these properties of the replacement construct or graft should be considered to ensure restoration of normal physiological function. It would also be interesting in the future to determine the mechanical and matrix properties of the human ACL and patellar tendon, as the ACL is currently repaired surgically using an autograft consisting of a graft from the patellar tendon or hamstrings, therefore it would be important to determine these properties of the replacement construct.

Our group are currently investigating whether age related changes to the mechanical and matrix properties occur in the human Achilles tendon based on findings in the horse model from the present study. Determination of the age at which tendon degeneration begins and the effect of exercise on the process are important areas to focus on in the future.

APPENDICES

APPENDIX I - Preparation of Chemicals

1. Transmission Electron Microscopy

- **Buffers**

0.1M Phosphate Buffer

Solutions A and B were prepared

A – Sodium Dihydrogen Orthophosphate (BDH Laboratory Supplies, Poole, UK)

0.2 M NaH_2PO_4 (anhydrous) 5.86g/200ml deionised water

B – Di-sodium Hydrogen Orthophosphate (BDH Laboratory Supplies, Poole, UK)

0.2 M Na_2HPO_4 (anhydrous) 1.56g/50ml deionised water

A 0.1 M solution was prepared by mixing A and B together, and making up to 500 mls with deionised water. PH 7.2. This was stored at 4°C for one month.

Phosphate-buffered 2.5 % Gluteraldehyde

10 ml 25 % gluteraldehyde (BDH Laboratory Supplies, Poole, UK) was mixed with 90 ml 0.1 M phosphate buffer, pH 7.2. This was stored at 4°C for one month.

- **Resin** (Spurr, 1969)

Component	Volume (g)
ERL 4206	20
DER 736	8
NSA	52
S-1	0.8

- **Lead Citrate** (Reynolds, 1963)

1. Mix 1.33 gms of $PBNO_3$ (lead nitrate) and 1.76 gms of $Na_3(C_6H_5O_7).H_20$ (trisodium citrate) in 30 ml deionised water.
2. Shake vigorously for 1 min. Allow to stand for 30 min with intermittent shaking to ensure complete conversion of lead nitrate to lead citrate.
3. Add 8.0 ml of NaOH, make up to 50 ml with deionised water and mix by inversion. Centrifuge if any turbidity is present. The pH is 12.0 ± 0.1
4. Store at 4°C. Stable for several months.

2. Histology

- **Paragon Multiple Stain**

30 % Ethanol	500 ml
Toluidine Blue	36.5 g
Basic Fuchsin	13.5 g

Mix all ingredients and stir for 30 minutes. This is a very saturated solution. This stain works better after is has 'aged' for about one month. Filter before use.

Staining slides

1. Place toluidine blue on slides to cover section and leave on for 10 minutes
2. Rinse slides in tap water and blot dry
3. Place paragon stain on slides for 15 minutes
4. Rinse slides in tap water and blot dry
5. Mount slide with DPX and coverslip

Results

Blue - nuclei, cartilage matrix and osteoid

Pink to Purple - mineralised bone, normal cellular cytoplasm and soft tissue components

3. Collagen Assay

- **Reagents**

Diluent

Propan-2-ol	100 ml
Water	50 ml

Stock Buffer

Sodium acetate (anhydrous)	6.87 g
Trisodium citrate.$2H_20$	7.5 g
Citric acid	1.1 g
Propan-2-ol	80 ml
Water	make up to 200 ml

Dissolve the solids in 100 ml water. Add the propan-2-ol and mix. Make up to 200 ml with water.

Oxidant

Chloroamine T	0.42 g
Water	5 ml
Stock buffer	25 ml

Dissolve the chloroamine T in water then dilute with stock buffer.

Colour Reagent

Dissolve the DMBA in the perchloric acid and then add propan-2-ol.

APPENDIX II - Processing Schedules

- **Histology**

Procedure	Time
10% Neutral Buffered Formalin	1 week
70% Ethanol	2 hours
90% Ethanol	2 hours
Absolute Alcohol (1st Change)	1.5 hours
Absolute Alcohol (2nd Change)	1.5 hours
Absolute Alcohol (3rd Change)	1.5 hours
Absolute Alcohol (4th Change)	Overnight
Absolute Alcohol (5th Change)	1.5 hours
L.R. White (1st Change)	1 hour at 4°C
L.R. White (2nd Change)	1 hour at 4°C
L.R. White (3rd Change)	Overnight at 4°C
Embed samples	

- **Transmission Electron Microscopy**

Procedure	Time
2.5% Gluteraldehyde in 0.1M Phosphate Buffer (cubed sample)	3-4 days
2.5% Gluteraldehyde in 0.1M Phosphate Buffer (fragments)	Overnight
Distilled Water	5 minutes
1% Osmium Tetroxide in Distilled Water	90 minutes
Distilled Water	Two 5 minute washes
70% Ethanol	Two 10 minute washes
90% Ethanol	Two 10 minute washes
96% Ethanol	Two 10 minute washes
Absolute Alcohol	10 minutes
Absolute Alcohol	20 minutes
Absolute Alcohol	30 minutes
1:1 Resin + Absolute Alcohol	90 minutes
100% Resin	2 changes of resin with vacuum infiltration (i.e. 30 minutes on rotar then 30 minutes in vacuum)
100% Resin change under vacuum	Overnight
100% Resin	2 changes of resin with vacuum infiltration
Embed samples into moulds, then placed into oven at 65°C	18-24 hours

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